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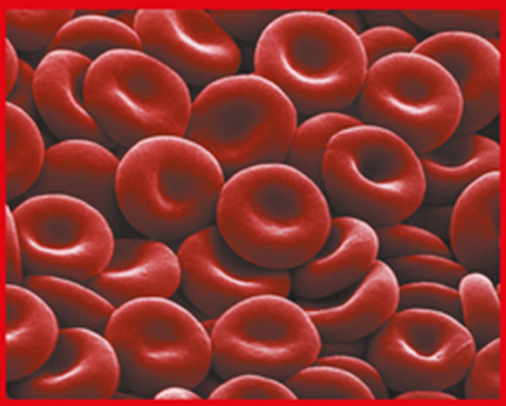
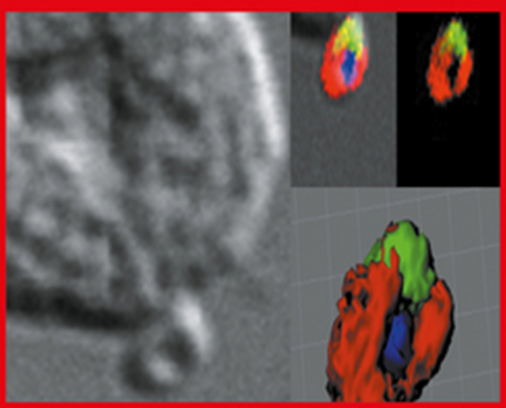
Advances in Malaria Research





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Deepak Gaur
Chetan E. Chitnis
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Advances in Malaria Research

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Advances in Malaria Research

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Cover Image:
Top Image: Red blood cells. Scanning electron micrograph of Red blood cells clearly showing their biconcave disc shape. (Source: Annie Cavanagh, Wellcome Images)

Middle Image: Plasmodium falciparum merozoite invading a red blood cell. Differential interference contrast (DIC) microscopy image of a merozoite attached to the red blood cell during invasion. The 3-D reconstruction of the confocal immunofluorescence image detected the leading vaccine antigen, PfRH5, at the apical end of the merozoite surface (Source: Reddy et al. PNAS USA 2015)

Bottom Image: Mosquito, Anopheles stephensi in flight. A mosquito (Anopheles stephensi) in flight with its abdomen full of blood. Through its role in the transmission of malaria and yellow fever, it is claimed that the mosquito has killed more than half the humans that have ever lived and it continues to kill between one and two million people a year, mainly young children. This particular species, Anopheles stephensi, is the insect vector that transmits malaria in India and Pakistan. (Source: Hugh Sturrock, Wellcome Image Award winner 2006)

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Dedicated to all our past and current students and group members

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Foreword

Malaria is still a major affliction of humans, despite the reported decreasing trend in incidence and mortality in recent years. It is reported that the incidence has reduced from around 500 million cases by almost half, and mortality has decreased from around a million to around 700,000, although the uncertainty range is rather high. This has been achieved through public health measures to protect against mosquito bites and by quicker diagnosis followed by appropriate drug therapies. But an effective vaccine is still not available, and the drugs, including artemisinin derivatives, are prone to become less effective because the parasite develops resistance to them. With a laudable ultimate goal to eradicate malaria, the challenges remain high, and this monograph entitled "Advances in Malaria Research", edited by Deepak Gaur, Chetan E. Chitnis and Virander S. Chauhan, is timely and captures the tremendous progress made in all facets of parasite biology.

The editors have assembled contributions from acknowledged experts to cover all facets of malaria research. Appropriately, the articles start with the process of mosquito injection of sporozoites and the development of the parasite in the liver stage, governing the pre-erythrocytic stage. This is followed by analysis of the molecular basis of erythrocyte invasion by *Plasmodium falciparum* and *Plasmodium vivax*. The next phase is obviously the transmission stage, and the next section of the book discusses rational and sustainable intervention options.

This is followed by a set of articles discussing the molecular basis of parasite metabolism. These include an analysis of the various layers of gene regulation, including epigenetic modifications and gene expression, covering all facets from transcription to post-translational modification. An understanding of the essential genes involved in erythrocyte-stage infection and adaptation to environmental changes with approaches such as conditional knockouts, followed by transcriptomic, proteomic, and metabolic pathway analysis, highlight the dynamic properties of the *Plasmodium* genome. The other molecular targets are the signal transduction pathways and transporters on the parasite membrane. At the molecular level, a detailed analysis of drug targets such as the parasite proteases and mechanisms of antimalarial drug resistance highlight the challenges involved in identifying new drug targets that can ultimately lead to drug molecules with ideal characteristics.

From the picture at the molecular level, the progression is toward an understanding of malaria pathogenesis based on the virulent features of the parasite. A major contributor to malaria pathogenesis is the host response influenced by host genetic factors and control of inflammation and acquisition of adaptive immunity. Drugs and vaccines have to be designed not only to combat falciparum malaria but also vivax malaria. *P. vivax* infection is no longer considered benign, with its unique properties for relapse long after treatment.

This monograph has been organized very logically in terms of an understanding of the challenges from the vector to the parasite and to the host in a seamless fashion, highlighting the current status in every stage of parasite development and disease manifestation. This is a volume that will be immensely useful to all malaria researchers and public health strategists.

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Preface

In the summer of 2011, Professor Angelo Azzi, the then-president of the International Union of Biochemistry and Molecular Biology (IUBMB) approached us to edit and compile a book on recent developments in malaria as part of the Wiley–IUBMB book series. This was an unexpected surprise, as we had never undertaken such an exercise before, and with great excitement we readily agreed. Angelo was eager to see a malaria book as part of IUBMB and Wiley's association to produce high-quality books on science that would have a strong impact on both researchers in the malaria field as well as students who seek the latest information on the subject. Malaria research has witnessed a boom since the turn of the century, with the advent of new technologies and concepts. Thus, the idea of having a book that reviews recent developments in malaria research was conceived. The three of us made a proposal for the book on the same lines to Wiley and IUBMB with a potential list of contributors who we hoped would join us in our endeavor. We are eternally grateful that almost everyone whom we approached with a request to contribute to our book readily accepted our invitation. Thus, we are proud to have 17 invited chapters from top-notch malaria researchers from the field, who have all been listed in the contributors section.

However, inexperienced as we were at the start of the project, little did we know what we were getting ourselves into. As is expected in these projects, some contributors sent their chapters well within time, whereas others, also our friends, had to be pursued by all means at our disposal. We badgered them regularly at the expense of potentially losing their friendship, soldiering on continuously, and have finally successfully compiled the malaria book.

Virander S. Chauhan (left), Deepak Gaur (center) and Chetan E. Chitnis (right)

We are delighted that coincidently this book commemorates 25 years of the establishment of the International Centre for Genetic Engineering and Biotechnology (ICGEB) New Delhi component, in which malaria research was taken up right at its inception and has remained one of its major flagship programs. Scientists in the malaria group work on different aspects of malaria research, including basic biology and drug and vaccine development, with a major focus on the erythrocytic blood stages of the malaria life cycle. What is unique about the ICGEB malaria program is that there is an equal focus on both *Plasmodium falciparum* and *Plasmodium vivax* malaria.

It is only befitting our work culture in the ICGEB malaria group that the three of us came together to edit and compile the book. We wish to specially acknowledge our group members, Enna Dogra Gupta, K. Sony Reddy, Tajali Sahar, Nidhi Hans, Chaitanya, Gaurav Anand, Hina Singh, Aakansha Kalra, Anushree Bhatnagar, and Alok Pandey, who helped us at the end in the final stages of formatting and compilations. We wish to put on record that all three of us thoroughly enjoyed reading all the contributions from some of the very best malaria researchers and honestly benefitted in our own understanding of malaria research. We sincerely hope that the students and researchers will also greatly benefit from reading this book, which contains the most updated state of-the-art information on malaria.

Deepak Gaur
Chetan E. Chitnis
Virander S. Chauhan
December 15, 2015

CHAPTER 1

Introduction: An overview of malaria and *Plasmodium*

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History

Malaria has threatened the human race for centuries. Ancient medical records from early civilizations based in India, China, and Mesopotamia have reported malaria as a disease characterized by intermittent fevers (Desowitz 1991; Cox 2002). In the fifth century BCE, the Greek physician Hippocrates classified the fever according to periodicity—*febris tertian* (every third day) and *febris quartana* (every fourth day)—and its association with splenomegaly (Cox 2002; Desowitz 1991; Pappas 2008). In the Middle Ages, the Romans coined the name *malaria* for the disease (Medieval Italian *mal,* "bad," and *aria* "air") because it was believed that the illness occurred due to toxic fumes and vapors arising from the marshy lands. This belief was further strengthened by the subsequent decline in malaria cases after the swamps were drained (Desowitz 1991).

It was only in 1880 that the true causative agent of the disease was identified when Alphonse Laveran (1845–1922), a military physician based in Algeria, first reported the crescent-shaped malaria parasites in the blood of a soldier suffering from intermittent fevers (Laveran 1881; Bruce-Chwatt 1981). He also observed motile filamentous structures emerging from a round spherical body, which he reported as appearing like an animal parasite. Laveran thus called this microscopic organism as *Oscillaria malariae* (Laveran 1881). Through his clinical examinations, he further observed that when he failed to detect the crescent structures there were no disease symptoms. He also observed that these microscopic organisms are cleared by quinine treatment.

Laveran's findings were further confirmed in 1885 by Ettore Marchiafava and Amico Bignami, who, using eosin-stained blood stains, also observed amoeboid movement of the organism (Marciafava and Bignami 1894). In 1886, Camillo Golgi was able to differentiate between tertian and quartan malaria and also defined the morphological differences of parasites responsible for the two types of malaria (Golgi 1886). He reported that the parasite underwent asexual reproduction and that fever was closely associated with lysis of the red cells and release of parasites. In 1890, Grassi and Feletti named the two different species *Plasmodium vivax* and *Plasmodium malariae* (Grassi and Feletti 1890). Sakharov (1889) and Marchiafava and Celli (1890) independently identified *Plasmodium falciparum* (Grassi 1900; Cox 2002; Cox 2010). Thus by 1890, it was known that malaria was caused by a protozoan parasite that invaded and multiplied in erythrocytes. Based on their

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periodic specificities and other characteristics, species specific for causing benign tertian (*Plasmodium vivax*), malignant tertian (*Plasmodium falciparum*), and quartan malaria (*Plasmodium malariae*) had been discovered (Cox 2010).

The next major question was: How does malaria transmission occur? This question was answered by the efforts of Sir Ronald Ross and Giovanni Battista Grassi. The idea that mosquitoes could transmit human disease first arose from the classical work of Sir Patrick Manson, who could be considered the father of tropical medicine and was also the mentor of Ross. In 1878, Manson was the first to demonstrate that a parasite (in this case the filarial worm) that causes human disease (elephantiasis) could infect a mosquito (Manson 1878). Ross, who was born in India, returned to India in 1895 and set about to prove the hypothesis of Laveran and Manson that mosquitoes were associated with malaria transmission.

Finally on August 20, 1897, in Secunderabad, Ross made his landmark discovery in which he observed the malaria parasite in the stomach of an *Anopheles* mosquito fed four days previously on a malaria patient, Husein Khan, who was suffering from intermittent fevers. Ross thus established the role of *Anopheles* mosquitoes in the transmission of human malaria parasites (Ross 1898). Ross was clearly on the verge of demonstrating the *Anopheles* mosquitoes to be responsible for human malaria transmission, but unfortunately he was unable to do so because at that stage he was transferred to Calcutta, a place with much less malaria.

He thus turned his attention to the avian malaria parasite, now known as *Plasmodium relictum,* which is commonly found in several bird species and which was a more convenient experimental model for malaria research. He discovered that the avian malaria parasite was transmitted by the gray (culicine) mosquito, *Culex fatigans*. Ross demonstrated malaria parasites in mosquitoes that had been fed on infected birds; the parasites developed and migrated to the mosquitoes' salivary glands, thus allowing the mosquitoes to infect other birds during subsequent blood meals. Thus, in 1897, Sir Ronald Ross elucidated the complete sexual-stage life cycle of *Plasmodium relictum* on the gut wall *Culex fatigans* (Ross 1898).

However, the actual evidence for the transmission of human malaria by *Anopheles* mosquitoes came from Bignami and Grassi in 1898, who had access to the malarial disease prevalent near Rome and Sicily. They showed that *Anopheles claviger* mosquitoes that fed on malaria-infected patients could via their bite transmit the disease to uninfected individuals (Grassi 1899). The Italians further went on to prove that it was only the female *Anopheles* mosquito that could transmit malaria, and they comprehensively described the blood stage mosquito life cycles of *P. vivax*, *P. falciparum,* and *P. malariae* (Grassi 1900). Later in 1899, Ross, during his posting in Sierra Leone, also demonstrated the development of the three *Plasmodium* species parasites in the *Anopheles* mosquitoes (Dobson 1999). *Plasmodium ovale* was discovered much later in 1918 by John Stephens (Cox 2010; Sutherland 2010).

Thus, the mode of malaria transmission through the *Anopheles* mosquito vector had been discovered and, in a great advancement to the field, provided a major method of protecting against the disease by reducing contact with the insect vector. The huge impact of this work was recognized when in 1902 Ronald Ross was awarded the Nobel Prize and in 1907 Charles Alphonse Laveran received the prize for establishing the role of protozoans as causative agents of human disease. In 1927, Julius Wagner-Jauregg was awarded the Nobel Prize for treating neurosyphilis by infecting patients with *Plasmodium vivax* (White 2011). This treatment was abandoned because it killed 15% of the patients. The fact that mosquito control was critical led to the development of several insecticides, including DDT, for whose discovery Paul Hermann Müller was awarded the Nobel Prize in 1948.

The complete life cycle of the *Plasmodium* parasites in humans was still not fully understood, especially the liver stages of development were not known at the time, and it remained a puzzle as to where the parasites resided for the first ten days after infection when they could not be observed in the blood. Although there were some suggestions that the parasites underwent another stage of

development besides in the blood, this was clouded for about four decades under the influence of a highly prominent German scientist Fritz Schaudinn, who in 1903 claimed the direct invasion of red blood cells by the infected *P. vivax* sporozoites (Schauddin 1903), even though these findings could not be confirmed. The first insight for the presence of an exoerythrocytic liver stage came from MacCallum's work on avian malaria: In 1898 he observed developmental stages of *P. relictum* in the liver and spleen of infected birds (MacCallum 1898). This was finally confirmed in 1947 by Henry Shortt and Cyril Garnham, who demonstrated that a stage of multiplication in the liver preceded the blood stages in the life cycle of the primate malaria parasite, *P. cynomolgi* (Shortt and Garnham 1948a). Thereafter, Shortt and Garnham detected exoerythrocytic liver forms of three *Plasmodium* species, *P. vivax* (Shortt 1948b), *P. falciparum* (Shortt 1949), and *P. ovale* (Garnham 1954) in humans. In 1986, Krotoski discovered the dormant liver stages known as hypnozoites that are characteristic only of *P. vivax* and are responsible for the relapse of malaria (Cogswell 1992; White 2011; Markus 2012).

The life cycle of *Plasmodium*

The above-mentioned research accomplishments led to our present-day understanding of the malaria life cycle that is common for all human *Plasmodium* species (Figure 1.1). The life cycle is initiated through the bite of an infected female *Anopheles* mosquito, which during a blood meal injects the spindle-shaped invasive stages known as sporozoites into the skin, from where they travel through the blood stream to the liver. In the liver, the sporozoite travels through multiple cells, including a Kuppfer cell, before finally residing in a hepatocyte. Within the hepatocyte, the single sporozoite grows and multiplies to yield around forty thousand invasive structures called merozoites. The merozoites are further released into the blood stream, where they invade red blood cells or erythrocytes. Within the erythrocyte, the parasite undergoes asexual reproduction known as schizogony, allowing a single parasite to produce 16 to 32 daughter merozoites, which, following egress, are released into the blood stream, leading to another cycle of erythrocyte invasion and growth.

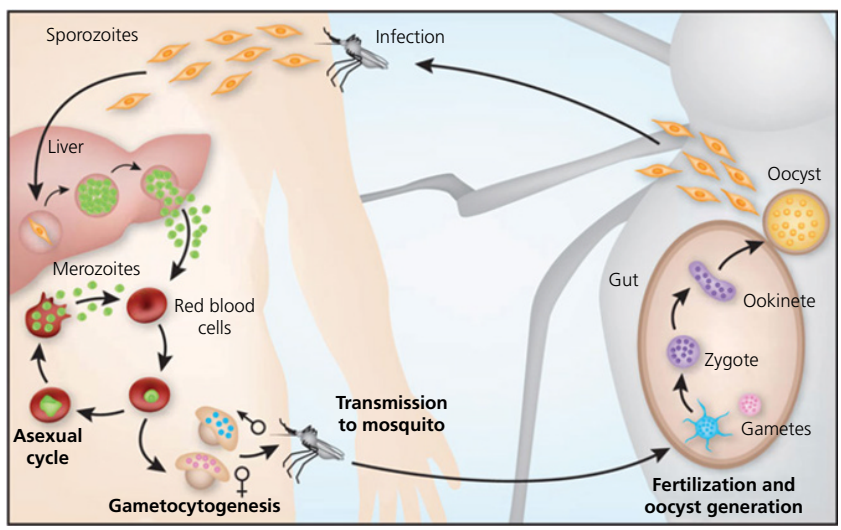

Figure 1.1 Life cycle of the malaria parasite. Image courtesy Geoffrey Pasvol (2010) Protective hemoglobinopathies and *Plasmodium falciparum* transmission. *Nature Genetics* 42: 284–285. (Nature Publishing Group).

It is the blood stages of the life cycle that are responsible for the clinical symptoms and pathology associated with the disease. The preceding exoerythrocytic liver stage does not produce any clinical symptoms, and in *P. vivax* infections, the hypnozoites could persist for decades without any symptoms. During the blood-stage life cycle, some parasites fail to progress and divide for reasons that still remain poorly understood. Instead, these parasites differentiate into gametocytes, which appear as crescent-shaped structures and were first observed by Laveran. During a blood meal, the male and female gametocytes are taken up by the *Anopheles* mosquito, the invertebrate vector host in which the sexual stage of the parasite's life cycle is completed. The male gametocyte forms motile microgametes that undergo exflagellation and swim to the macrogamete formed from the female gametocyte. The microgamete fertilizes the macrogamete resulting in the formation of the motile zygote, known as the ookinete, which traverses the epithelial lining of the midgut and finally resides in a thick-walled structure known as the oocyst under the mosquito's outer gut lining. Within the oocyst, several sporozites are formed through asexual multiplication and make their way to the mosquito's salivary gland. It is these sporozoites that get injected in to the human host through the bite of the mosquito, thus completing the life cycle that progresses from there.

A significant milestone in malaria research: Adaptation of *Plasmodium* to laboratory culture

It has been more than a century since Ross's landmark discovery, and during this ensuing period, the first fifty years of research on malaria parasites were focused on describing the parasite, identifying it as the causative agent of malaria, understanding its mode of transmission, and uncovering the different stages of its complex life cycle. A major obstacle during this period was the difficulty in culturing the parasite *in vitro* in the laboratory, due to which primary experiments were performed with primate models such as *Aotus* monkeys and even human volunteers. Initial attempts to culture *Plasmodium* parasites failed because the cultures were always short term, in which the parasite numbers were constantly decreasing till they completely died.

The continuous culture of *P. falciparum* was successfully established by William Trager and James Jensen in 1976. The novelty of their work was to try to culture the *P. falciparum* clone FVO obtained from an *Aotus* monkey under low oxygen concentrations (5% O_2/7% CO_2/88% N_2) with commercial RPMI 1640 medium in flow vials with a settled layer of human red cells (Trager and Jensen 1976). In these conditions, Trager and Jensen were able to culture the parasites for 24 days with timely addition of fresh red cells every 2 to 3 days. Another innovative development from them was to employ an old microbiological candle-jar method that was historically used for anaerobiosis to generate a CO_2-rich atmosphere. This simple methodology made it much easier to culture malaria parasites even in remote settings that did not have access to mixed gases (Trager and Jensen 1976).

Whereas Trager and Jensen were the first to report the continuous culture of *P. falciparum* in their *Science* paper published in August 1976, another group, led by J. David Haynes, also demonstrated the same findings, which were published the same year in the journal *Nature* in October (Haynes 1976). Haynes demonstrated that in addition to human red cells, chimpanzee red cells also supported *P. falciparum* growth but red cells from rhesus monkeys and guinea pigs did not (Haynes 1976). The present generation of malaria researchers might find it difficult to envisage a time when *P. falciparum* culture was not possible because it is such an important and integral part of current daily efforts in malaria research. Thus, it is difficult to gauge the enormous impact of continuous *P. falciparum* culture cultivation on malaria research across the world.

Probably no other single development has had such a profound and unparalleled influence on malaria research as observed with the laboratory culture of malaria parasites. It completely

revolutionized malaria research and enabled studies on different aspects of malaria that are being pursued with great vigor even today in fields include biochemistry and molecular and cellular biology of the parasite; host–parasite interactions especially, with human red cells; immunology and vaccine development; drug development and resistance; pathogenesis; gametocytogenesis and mosquito transmission; and genetics of the parasite and red cell susceptibility. Basically, malaria parasite culture brought accessibility to scientists who otherwise did not have access to clinical malaria cohorts and thus allowed an astounding expansion in malaria research. Overall, the successful *in vitro* culture of *P. falciparum* remains the basis of present-day malaria research.

The advent of present-day technologies and their applications in malaria research

Malaria research has benefited immensely with the onset of new innovative technologies, high-throughput platforms, and novel biochemical and molecular approaches, which have led to a boom in major discoveries that have unraveled the intricacies of the malaria parasite at a molecular level. The past couple of decades have seen the growth of -omics technologies that have been vigorously applied to malaria parasites. First was genomics, which with the help of advanced sequencing techniques and instrumentation allowed the sequencing of various pathogens including *Plasmodium* species. The publication of the complete sequence of the *P. falciparum* genome in 2002 (Gardner 2002) was a landmark accomplishment that was followed by the genomes of *P. vivax* (Carlton 2008), *P. knowlesi* (Pain 2008), *P. cynomolgi* (Tachibana 2012), *P. yoelii* (Carlton 2002), *P. berghei* (Hall 2005), and that of the *Anopheles* mosquito vector (Holt 2002). The genome provided a complete map of all possible genes and noncoding regions of the parasite that greatly benefited molecular studies.

This was followed by transcriptomics, which provided the description of the transcriptome of *P. falciparum* (Le Roch 2003; Bozdech 2003) and later *P. vivax* (Bozdech 2008) through the application of high-throughput microarrays. The transcriptome provided a timeline for the expression of gene transcripts during the complete 48-hour intraerythrocytic life cycle. These studies provided a blueprint that showed how regulation of gene expression in the malaria parasite was so tightly controlled and sparked further research to identify transcription factors and understand gene regulation in *Plasmodium*.

The transcriptome was complemented at the level of both protein translation and metabolites by proteomics and metabolomics (Johnson 2004; Hall 2005; malERA Consultative Group 2011). Advances in proteomics and metabolomics were set in motion with the advent of new-generation, highly sensitive mass spectrometry that could detect molecules and their modifications with great precision and accuracy. These data, together with development of methods for genetic manipulation of malaria parasites, have made it possible to probe the function of individual genes to understand the biology of these pathogenic organisms.

Research on malaria is a wide field that encompasses multiple disciplines. These include clinical research, epidemiology, and translational research toward development of new drugs and vaccines at one end and reductionist approaches to study the molecular and cell biology of malaria parasites at the other end of the spectrum. Often, to address problems in malaria effectively, there is a need to combine approaches such as the application of molecular approaches to studies in the field. This book covers the whole range of such topics to provide a snapshot of our current understanding of malaria in all its diverse aspects. It provides an update on our understanding of the biology of malaria parasites at different life cycle stages, their interaction with the host and vector, the epidemiology of malaria, and the progress made in the development of novel prophylactic and therapeutic

strategies against malaria. While remarkable progress has been made, there are still significant gaps in our knowledge. This book points out these gaps and suggests new directions to address these unsolved problems. There is a need to continue our efforts to improve our understanding of malaria, which is key to developing novel strategies to control, eliminate, and, hopefully, eradicate malaria in the future.

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CHAPTER 2

Exoerythrocytic development of *Plasmodium* parasites

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The concept of exoerythrocytic development of mammalian *Plasmodium* species goes back to 1900, when Grassi postulated that sporozoites do not directly infect red blood cells (Grassi 1900). However, in 1903, Schaudinn reported that *Plasmodium vivax* sporozoites enter erythrocytes and suggested a direct development of erythrocytic forms from sporozoites (Schaudinn 1903). This incorrect interpretation by a leading parasitologist of the time misled the field for almost 30 years before his hypothesis was challenged by the observation that only *P. vivax* sporozoite inoculation, and not the inoculation of infected blood, resulted in relapses and that the blood- and sporozoite-induced infections differed in their responses to quinine (James 1931; Korteweg 1931; Van Assendelft 1931). In 1931, van Assendelft postulated that initial exoerythrocytic development would occur at the site of the mosquito bite (Van Assendelft 1931), but this was disregarded from 1948, when Garnham discovered schizonts of *P. cynomolgi* and *P. vivax* in the liver of monkeys and human volunteers, respectively (Garnham 1948). Only very recently, Gueirard and coworkers demonstrated that *Plasmodium* sporozoites can indeed develop in skin cells (Gueirard 2010), giving some overdue credit to van Assendelft's 80-year-old hypothesis.

A clear breakthrough in *Plasmodium* liver stage research was the identification of rodent *Plasmodium* species that could be established as robust model systems to investigate the exoerythrocytic phase in the life cycle of the parasite. However, it took a very long time from the discovery of *Plasmodium berghei* in 1948 (Vincke and Lips 1948) until structured liver stage research was initiated. The main problem was producing infectious mosquitoes in a standardized way, and this was finally solved in 1964 when Yoeli and coworkers established the correct culture conditions for infected mosquitoes (Yoeli 1964). Despite this, it took until the 1980s before the entire exoerythrocytic development was examined at an ultrastructural level and presented in a number of publications that are discussed in a famous and very complete review on exoerythrocytic development of malarial parasites (Meis and Verhave 1988). At this time, techniques were mainly restricted to light microscopy and electron microscope (EM) analysis, and these scientists provided us with an impressive series of EM images and, by very careful observation and interpretation of the data obtained, drew correct conclusions that shed light on this former black box in the life cycle of mammalian *Plasmodium* parasites.

Although recent years have revealed many molecular details about this parasite stage, the major findings of Meis and the other colleagues at this time still hold true. More than 25 years after their original description of the exoerythrocytic development of rodent and human *Plasmodium* species, the ultrastructure images still provide us with important information and help explain new findings

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and, if carefully reinvestigated in view of our current knowledge, may even generate new hypotheses. This chapter is therefore a tribute to these scientists and their highly appreciated work on the exoerythrocytic stage of *Plasmodium* parasites. We also try to cover the flourishing field of *Plasmodium* liver stage research of recent years and put the findings in context of the observations made more than two decades ago. It is, however, impossible to discuss all the valuable work on the exoerythrocytic development of malaria parasites from the past and the present, and we apologize to all the authors not mentioned in this chapter.

We will first concentrate on the fate of the parasite from injection by the mosquito until egress from hepatocytes before discussing some theories postulated 25 years ago and now proved correct by molecular techniques and advanced microscope techniques.

The sporozoite's journey from the skin to the liver

For many years one of the least-understood phases of infection was the migration of sporozoites in the mammalian host because it was, and still is, very difficult to experimentally approach this. Initially, it was thought that the mosquito injects sporozoites directly into a blood vessel and that the parasites are then transported to the liver, where they pass through gaps in the endothelial cell layer to reach hepatocytes. We now know that during a blood meal, mosquitoes do not probe a blood vessel but instead cause a hematoma in the tissue and suck the blood from this area (Matsuoka 2002; Sidjanski and Vanderberg 1997; Frischknecht 2004). During this blood meal, sporozoites (Figure 2.1) are injected into the skin through a salivary duct within the proboscis of the female mosquito. One can imagine a proboscis as a mantle around the actual food channel and the salivary duct. The main function of the salivary duct is to inject saliva into the skin to locally anesthetize the skin and to cause the aforementioned hematoma. Sporozoites take advantage of this injection and are delivered together with the saliva into the skin. From there they have to find their way to the blood vessel (Frischknecht 2004). To migrate in the skin, sporozoites use gliding motility that is based on an actin–myosin motor (Amino 2006; Kappe 1999) anchored in the inner membrane complex (IMC) (Matuschewski and Schuler 2008) (Figure 2.1). The connection to the surface on which the sporozoites glide is made by secreted adhesion molecules like CSP (circumsporozoite protein) and TRAP (thrombospondin-related anonymous protein).

Because reaching a blood vessel occurs by chance rather than by chemotaxis, only a fraction of sporozoites finally arrive at their destination, whereas the majority do not leave the skin, or they enter lymph vessels and reach the draining lymph node, from where they are eventually removed (Amino 2006). It has been shown that *P. berghei* sporozoites that remain in the skin can fully develop to infectious merozoites inside skin cells (Gueirard 2010). This finding gave credit to the hypothesis of van Assendelft, who suggested an exoerythrocytic phase of the parasite in the skin based on morphological differences of the sporozoites and blood stage merozoites (Van Assendelft 1931). However, even if *Plasmodium* sporozoites can infect a wider range of cells than originally thought (Gueirard 2010), hepatocytes are the main cell type they invade to continue their development. On their way to infect hepatocytes, there are two major hurdles for the parasites: firstly they need to recognize the liver, and secondly they have to cross the endothelium of the blood vessel to gain access to hepatocytes.

Sporozoites reach the liver by passive transport in the blood stream and are able to probe the environment because of the low speed of blood flow in liver sinusoids. Only in such an environment are they able to adhere to the endothelium and to bind to highly sulfated heparin sulfate proteoglycans (HSPGs) (Coppi 2007) presented by hepatocytes through small channels in the endothelium, called fenestrae (Figure 2.2A). These fenestrae were already discussed by Meis and Verhave as a possible

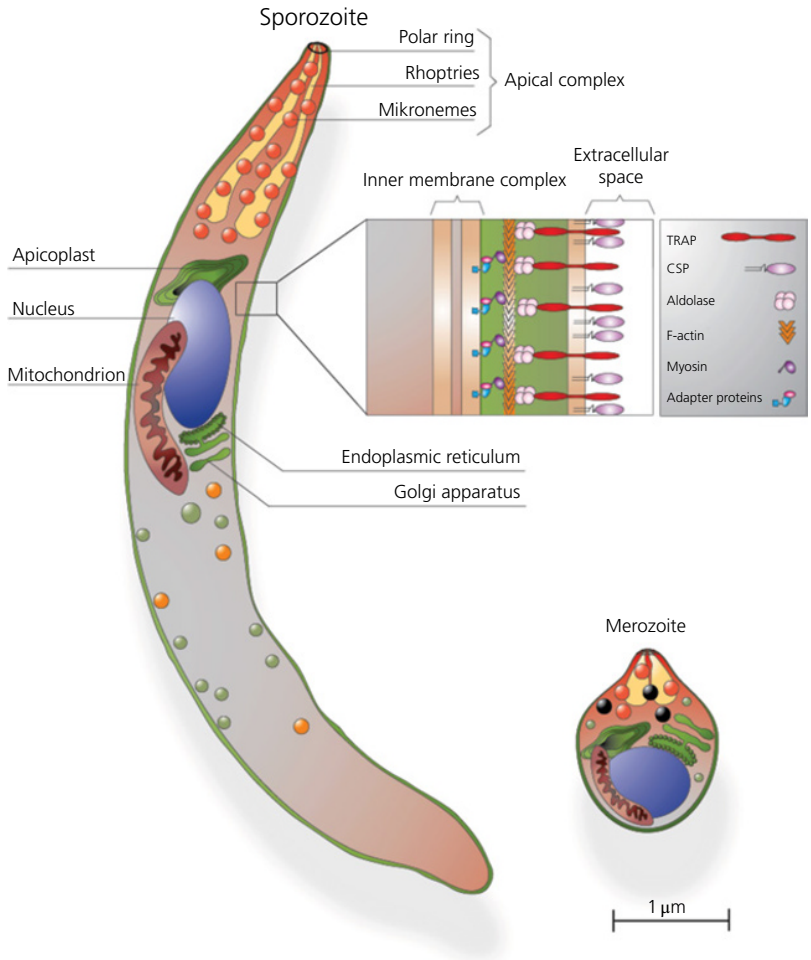

Figure 2.1 Comparison of the two *Plasmodium* invasive forms in the mammalian host. On the left, a sporozoite is depicted, and on the right, a merozoite is shown. For the sporozoite, the composition of the membrane and underlying features, including the molecules responsible for gliding motility, are shown as a magnified image.

route of entry to hepatocytes, but because they are about 10 times narrower (0.1 µm) than the diameter of a sporozoite (1 µm), this hypothesis was rejected (Meis and Verhave 1988). Although the parasite receptor for HSPGs has not yet been identified, we now understand rather well the molecular events following sporozoite binding to HSPGs, when the parasite switches from the migration mode to the invasion mode.

The main sporozoite surface protein, CSP, plays a major role during this switch (Coppi 2011). In the migration mode, the N-terminus of CSP masks the C-terminal cell-adhesive thrombospondin repeat (TSR) domain. Upon binding to HSPGs, the C-terminus is proteolytically cleaved off. CSP processing and exposure of the TSR allows the parasite to firmly attach to the endothelium and must be a very well coordinated event. A possible regulator of CSP processing is the cysteine protease inhibitor ICP (for inhibitor of cysteine proteases) (Hansen 2011; Rennenberg 2010). In *P. berghei* this inhibitor is called PbICP; in *Plasmodium falciparum* it is called falstatin (Pandey 2006). It is expressed

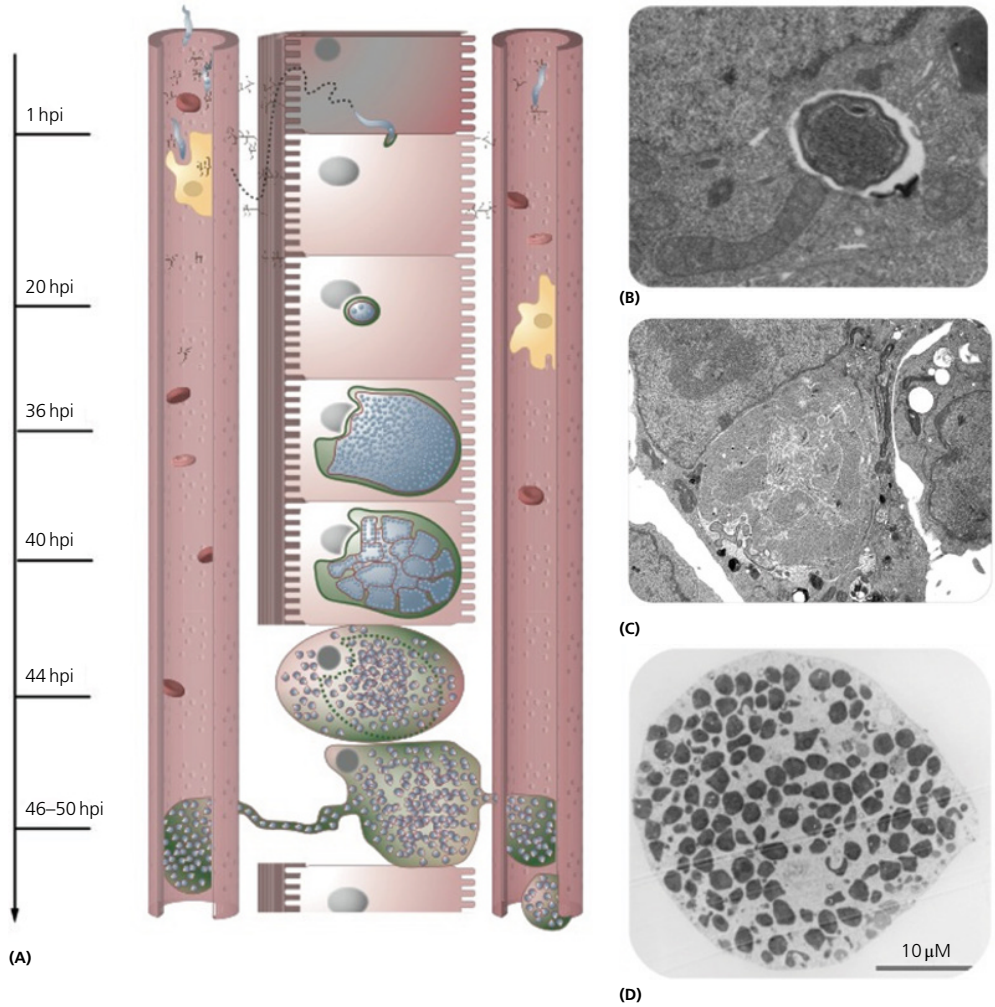

Figure 2.2 Exoerythrocytic development of *Plasmodium* parasites. *A,* On the left, a time scale of the events is shown. The cartoon shows the entire development from transmigration of the sporozoites to budding of merosomes. On the right, three TEM images are shown. *B,* Cross section of an invaded sporozoite. *C,* Developing schizont. *D,* Merosome containing many merozoites. (Image from Heussler, 2012.)

throughout the life cycle of *Plasmodium* parasites, suggesting that it has various functions. Sporozoites secrete PbICP, and this might control CSP processing, which is indeed mediated by a cysteine protease once the sporozoites come into contact with hepatocytes (Coppi 2011).

Sensing the correct environment in liver sinusoids by HSPG binding and attaching to the endothelium is not, however, sufficient; the parasite must still cross the endothelium to reach its final destination, the hepatocytes. Early transmission electron microscope (TEM) and scanning electron microscope (SEM) studies suggested the existence of small intercellular gaps in the endothelium of liver sinusoids, large enough to allow the passage of sporozoites. This observation easily explained how parasites could pass through the endothelium. However, later it was shown that these gaps are probably fixation artifacts, and a new explanation was needed (Meis and Verhave 1988). An alternative hypothesis was therefore that sporozoites transmigrate through cells of the endothelium.

To prove this hypothesis was extremely difficult because studies were again restricted to EM analysis. To identify the tiny sporozoites inoculated by mosquitoes or via a needle during a very brief period of transmigration by ultrastructural analysis of infected rodent livers must have been an extremely time consuming and sometimes frustrating process.

To increase the numbers of infected cells, Meis and Verhave used the highly susceptible Brown Norway rat strain and additionally ligated the blood vessels of the rat liver in a way that concentrated exoerythrocytic stages in the smaller right and caudate lobes (Meis and Verhave 1988). Today the problem of identifying infected hepatocytes could potentially be overcome by employing Correlative Light Electron Microscopy (CLEM), a technique that is based on the identification of fluorescent features (for example genetically modified fluorescent parasites) by light microscopy in an epoxy resin, allowing the trimming of the block to yield sections of the identified feature and subsequently very focused ultrastructural analysis. In the 1980s, genetic manipulation of *Plasmodium* parasites had not yet been established, and so Meis, Verhave, and others must have screened thousands of EM images before providing the first systematic analysis of sporozoite migration through the endothelium. They identified sporozoites passing through Kupffer cells (specialized resident macrophages of the liver) to reach the Space of Disse and the adjacent hepatocytes (Meis 1983; Meis 1985a). This observation was surprising because Kupffer cells are professional phagocytes and are thought to eliminate migrating sporozoites. The concept of sporozoites passing through Kupffer cells was supported by the results of experimental infection of silica-treated rats, in which the phagocytic ability of Kupffer cells is impaired (Smith and Sinden 1982; Verhave, Meuwissen, and Golense,1980). Infection rates of such treated animals were significantly reduced. It was also shown that viable sporozoites resist the oxidative burst of Kupffer cells, in contrast to heat-killed sporozoites, which induce the respiratory burst (Smith and Alexander 1986).

More recently, by intravital imaging of fluorescent parasite strains, it has been shown how sporozoites glide along the sinusoid endothelium and then pass through a constriction in the endothelium to access the liver parenchyma (Amino 2008; Frevert 2005; Frischknecht 2004). Similar studies have confirmed the initial observation of Meis that sporozoites can indeed pass through Kupffer cells (Pradel and Frevert 2001), but it still has to be shown whether or not this is the only route that sporozoites use to leave the blood vessel. Because they do not need Kupffer cells or other macrophage-like cells to transmigrate endothelia in the skin, alternative routes might exist.

Sporozoites can transmigrate through cells in different ways. One is an aggressive wounding of the cell membrane during entry and exit, which leads to the release of some intracellular material (Mota 2001). Cell wounding and release of cell content may in turn attract phagocytes to the site of transmigration. A second transmigration route has been described for sporozoites passing through Kupffer cells (Pradel and Frevert 2001). It involves an invagination of the host cell plasma membrane at the point of entry and likely a membrane fusion event at the exit site of the transmigrated cell. Immune cells employ a similar mode of transcellular migration to rapidly cross endothelia to reach the site of infection (Muller 2010). They pass through a cell in a membranous channel without injuring the traversed cell. Which method of transmigration sporozoites use *in vivo* to reach hepatocytes remains to be shown, but the less-aggressive method of membrane invagination and fusion could be advantageous to the parasite because it is immunologically silent. The observation that sporozoites squeeze through a single constriction in the membrane of the transmigrated cell (Amino 2008) fits better with the membrane-invagination and membrane-fusion model than with the wounding model, considering that for the latter, one would predict two parasite-constriction events, one upon entry and one upon exit.

Knockout studies suggested a number of proteins involved in transmigration (Ejigiri and Sinnis 2009; Ishino 2004; Ishino 2005) but the exact molecular events are not understood yet. Once sporozoites have crossed the endothelium, several parenchyma cells are also transmigrated before

the parasite finally switches into invasion mode (Amino 2008; Coppi 2011; Mota 2002). Most likely this switch is a progressive event mediated by proteolytic processing of CSP, resulting in the exposure of adhesive domains. It might be that invasion only happens if the majority of CSP is processed and little newly produced full-length CSP is present. Still it remains to be solved how the parasite induces host cell membrane invagination once it has left the last transmigrated cell. It is very difficult to imagine how this would happen if the transmigration occurs by cell wounding. Considering that the membranes of neighboring cells are in very close proximity, how can the sporozoites sense the membrane of the hepatocyte to be invaded? If, however, transmigration occurs instead by the aforementioned silent transmigration, with sporozoites forming channels through hepatocytes, it is easy to imagine that invasion and transmigration are similar processes, with the only difference that during transmigration the membrane at the exit site must fuse to allow escape of the parasite. Future research will hopefully shed some light on these events because for intervention strategies it is important to know if transmigration is receptor-mediated and immunologically silent or due to cell-wounding causing inflammatory immune responses.

Sporozoite invasion

Whichever mode sporozoites use to transmigrate through sinusoidal endothelia and hepatocytes, finally they invade a hepatocyte by the formation of a parasitophorous vacuolar membrane (PVM) (Figure 2.2B). The major breakthrough in the analysis of sporozoite invasion was the establishment of an *in vitro* infection system for rodent *Plasmodium* species (Hollingdale 1981; Hollingdale 1983a; Hollingdale 1983b). It turned out that *in vitro*, in addition to primary hepatocytes, *P. berghei* can infect a wide range of cells including hepatoma cell lines of mouse and human origin, as well as cell lines of nonhepatocyte background like HeLa cells and fibroblasts. It is well known that the *Plasmodium*-related parasite *Toxoplasma gondii* has a broad spectrum of hosts and within these hosts can infect a wide range of differentiated cells. To achieve infection of so many different cell types, *T. gondii* injects rhoptry neck (RON) proteins, such as RON2, into the host cell that than serve as receptors on the host cell plasma membrane for parasite surface proteins like AMA1 (Besteiro 2011). Both types of proteins are well conserved among apiciomplexan parasites, and it has been suggested that they have a similar function also in *Plasmodium* zoites (Ejigiri and Sinnis 2009). Although this concept is indeed very attractive, it still remains to be shown how the sporozoites make the initial contact with their final host cells. Perhaps a number of receptors are involved in forming low-affinity attachment. It has indeed been shown that *Plasmodium yoelii* and *P. falciparum* sporozoite invasion depends to a certain extent on the expression of CD81 and SRBI (scavenger receptor, class B, type I) receptors on host cells (Rodrigues 2008; Silvie 2003; Yalaoui 2008). However, it appears that other receptors might also be involved because blocking the known receptors reduces but does not abolish sporozoite invasion, and *P. berghei* seems not to rely on CD81 and only partly on SRBI receptors.

It also remains to be solved how different parasite proteins are secreted in the highly ordered processes of sporozoite attachment to host cells and invasion. Rhoptry neck proteins are among the first-line proteins to be discharged, but other vesicles like micronemes or exonemes might be also involved (Figure 2.1). It would also be important to determine how transmigrating sporozoites differ from invading sporozoites in terms of protein secretion. The content of the discharged vesicles most likely includes proteases because it has been shown that externally added protease inhibitors can block invasion (Coppi 2005; Kumar 1994). On the other hand, protease activity needs to be tightly controlled, and indeed sporozoites express a protease inhibitor that is partly stored in micronemes and is constitutively secreted by the parasite (Rennenberg 2010). Interestingly, neutralization of PbICP inhibition by

a specific anti-PbICP antiserum resulted in a significant reduction of sporozoite invasion, and the generation of conditional PbICP knockout parasites has confirmed this impressively. PbICP knockout parasites are completely unable to invade hepatocytes (Lehmann 2014). What could be the target protease, and even more importantly, which protein is processed by the target protease if the inhibitor is not present? An obvious target protease is the so-far-unknown cysteine protease that is needed to process CSP, and indeed PbICP knockout parasites exhibit a premature CSP processing phenotype (Lehmann 2014). This would explain the observation that PbICP knockout sporozoites are unable to glide, but it does not explain why they cannot invade hepatocytes. Most likely the protease regulated by PbICP has many targets other than CSP and therefore induces this drastic phenotype.

Sporozoite invasion of hepatocytes is accompanied by membrane invagination, but information regarding modulation of the host cell membrane by the parasite is only starting to emerge. For a long time it was assumed that the sole driving force behind invasion is the gliding motility of the sporozoite, but it has been shown that host cell actin also plays an important role in sporozoite invasion (Gonzalez 2009). However, sustained actin polymerization around the developing parasite appears to contribute to parasite elimination (Gomes-Santos 2012). Interestingly, the same is true for the autophagy marker protein LC3, another host cell protein that is incorporated into the PVM briefly after sporozoite invasion (Prado 2015). Although the PVM is of host cell origin, it is generally accepted that the majority of host cell proteins are removed during the invasion process, most likely by proteases secreted by the parasite into the forming vacuole and that the membrane is heavily remodeled by the parasite to suit its needs.

There is no doubt, however, that the host cell recognizes the invading parasite and tries to isolate it by LC3 incorporation into the PVM (Prado 2015). LC3-labeling of membranes is followed by lysosome fusion, and it has been suggested that in *P. berghei*–infected hepatocytes, lysosomes indeed fuse with the PVM and contribute to nutrient and membrane supply for the growing parasite (Lopes da Silva 2012). If this is true, how does the parasite avoid acidification of the PV? One suggestion of these authors is that only early lysosomes that still have a moderate pH fuse with the PVM, and thus the pH in the vacuole does not significantly drop. Another explanation is that generation of a pH gradient needs a membrane that does not allow the free flow of H^+ ions. It is known, however, that the PVM allows the passage of molecules of up to 700 Da, and thus it is unlikely that a pH gradient can be generated.

All together, the following concept might apply to *Plasmodium* sporozoite invasion in hepatocytes: Sporozoites make first contact in the liver sinusoids with HSPGs presented by hepatocytes, and CSP processing is induced. Sporozoites transmigrate the endothelium and make contact with a number of low-affinity receptors on the host cell surface. This induces protein secretion from the rhoptries, and RON2 among others is injected into the host cell. RON2 associates with host cell actin and is presented on the host cell membrane as a receptor for AMA1, which is located on the surface of sporozoites. This finally results in membrane invagination and the formation of a PVM, and the parasite is pulled into the host cell supported by a host cell actin–myosin motor.

Parasite development

Surrounded by a parasitophorous vacuole and within its host hepatocyte, the exoerythrocytic *Plasmodium* parasite undergoes an expansive growth and a rapid replication. *In vivo* intracellular liver stage parasites can grow to a size bigger than the original host cell, and they can form up to 30,000 merozoites from a single invading sporozoite, depending on the *Plasmodium* species (Meis and Verhave 1988). Growth and replication on this scale is no mean feat. To achieve this, the

parasite needs to undergo several transformation steps and to explore host cell resources without inducing adverse events in the host cell, such as apoptosis or separation in a degradation vacuole. Nutrient transport from the host cell to the parasite and signaling from the parasite to the host cell is complicated by the fact that the parasite resides in a parasitophorous vacuole. Parasite proteins need more than a simple signal sequence to reach the host cell cytoplasm, and some possibilities of how this protein transport might be achieved are discussed below. Other topics in this chapter are the modification of the PVM to support nutrient supply and protein transport and how the parasite subverts the host cell signaling pathways to guarantee its own survival. First, however, we discuss parasite transformation from the crescent-shaped sporozoite to a multinuclear schizont.

While progress on studying exoerythrocytic development lagged behind that of other stages due to the complexity of generating and examining infected hepatocytes, relatively recent advances in parasite culture and in microscopy have shed light on various aspects of exoerythrocytic development. It is important, however, to note that many key features of the development were observed and described some decades ago.

The crescent-shaped sporozoite, having invaded a hepatocyte, must transform to form a rounded trophozoite. It has been known since the 1980s that sporozoites rapidly localize beside the host cell nucleus (Aikawa 1984; Meis and Verhave 1988; Suhrbier 1986), and more recent studies have shown this to be beneficial for development, with parasites close to the nucleus being larger than those further away (Bano 2007; Graewe 2012). The significance of this interaction is not yet clear, and neither are the mechanisms that allow the parasite to reach this perinuclear position. It is speculated that a position close to the host cell nucleus and hence close to the Golgi apparatus may allow hijacking of vesicular material en route from the host endoplasmic reticulum to the Golgi apparatus (Figure 2.3).

Observations of the transforming or dedifferentiating sporozoite from the 1980s described the shape changes undertaken by the sporozoite as it develops into a trophozoite (Meis 1983; Meis and Verhave 1988). These observations have been added to by work from Jayabalasingham and colleagues (Jayabalasingham 2010). Following invasion, the sporozoite initially maintains a crescent shape, retaining its invasive morphological features such as micronemes, rhoptries, and subpellicular microtubules (Meis and Verhave 1988). At approximately 2 to 4 hours after invasion of the hepatocyte, the sporozoite forms what is commonly known as a belly, a protrusion halfway along its length and close to the nucleus. This bulb increases in diameter over time (Jayabalasingham 2010), and the distal ends of the sporozoite retract. The bulbous region has been seen by SEM to have a wrinkled surface, indicating indentation of the plasma membrane at this site. The bulbous belly region appears to expand at a site where "inner membranes" (Meis and Verhave 1988), now known to be the inner membrane complex, are lost from beneath the plasma membrane. The inner membrane complex, flattened membranous sacs that provide rigidity to the motile parasite and the anchor of the parasite's actin–myosin motor, underly the plasma membrane of each motile stage of the *Plasmodium* life cycle. A similar transformation undertaken by the ookinete as it rounds up to form an oocyst may well also result from the removal of the inner membrane complex at this stage. Ookinete-to-oocyst transformation also occurs by formation of a central bulbous region followed by retraction of distal ends.

Disruption of the sporozoite's inner membrane complex by knockout of the protein IMC1a in *P. berghei* leads to premature formation of the bulbous region and a lack of sporozoite motility (Khater 2004). It has been speculated that the IMC destruction may be the result of autophagy, a process by which a cell degrades its own material in order to provide nutrients in periods of starvation or to allow remodeling (Jayabalasingham 2010). Components of an autophagy machinery have been identified in the *Plasmodium* genome, but thus far localization studies have shown proteins to be associated with the apicoplast and mitochondrion rather than to autophagic vesicles as seen in other systems (Eickel 2013; Kitamura 2012). Osmiophilic whorls were observed in the

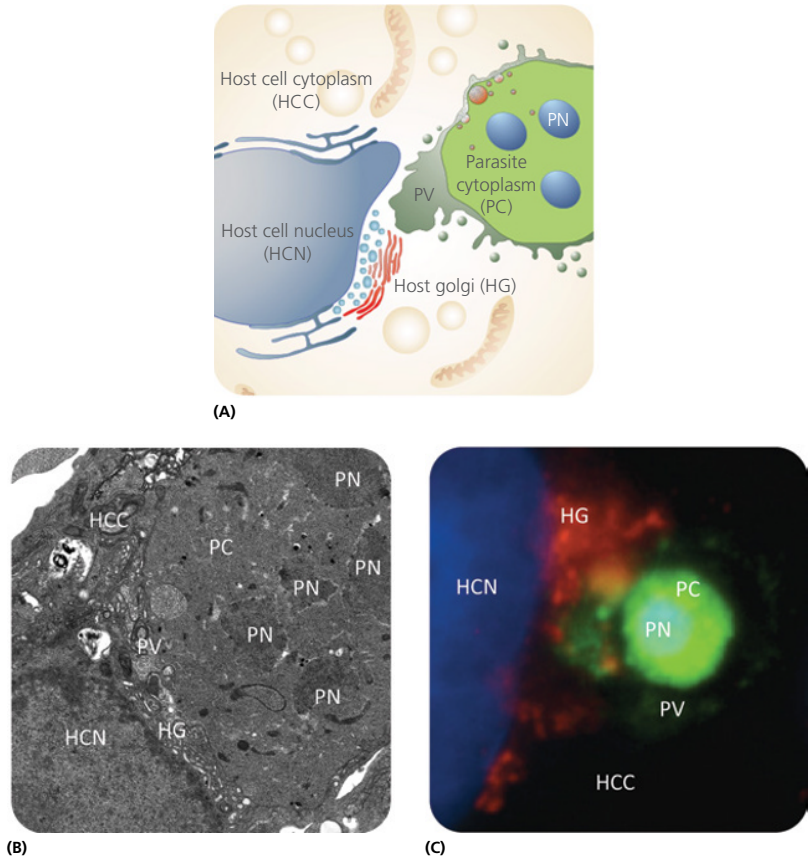

Figure 2.3 *P. berghei* schizont develops close to the host cell Golgi. A schematic representation *(A)*, a TEM image *(B)*, and an IFA image *(C)* are shown. HCC: host cell cytoplasm; HCN: host cell nucleus, HG: host Golgi; PC: parasite cytoplasm; PN: parasite nucleus; PV: parasitophorous vacuole. For the IFA, HepG2 cells were infected with *P. berghei* sporozoites and fixed 16 hpi. Staining of the host-cell Golgi was with an anti-BIP antibody *(red)* and the parasite was stained with an anti-PbICP antiserum *(green)*. DNA was visualized with DAPI. (*See insert for color representation of this figure*.)

parasitophorous vacuole (PV) space surrounding the transforming sporozoite and were believed to be rhoptry secretions (Meis and Verhave 1988), but others speculated that this may be IMC material packaged and excluded from the parasite into the PVM, an alternative method used by some cells to dispose of unwanted or deleterious material (Jayabalasingham 2010). Reports have described the apical complex of the invasive sporozoite to still be visible 15 hours after infection before being lost (Meis and Verhave 1988), and it has been suggested that micronemes may be gathered into membrane-bound packages and extruded by the transforming sporozoite into the PV (Jayabalasingham 2010).

The sporozoite continues its transformation by losing its distal ends. It is important to note that the process of sporozoite transformation to the round trophozoite can be followed axenically without host cells, demonstrating that the host hepatocyte is not required for these initial transformation steps (Kaiser 2003). Axenic sporozoite transformation, however, occurs more slowly than development within the hepatocyte, implying that the host cell provides some advantages as a site for dedifferentiation of the parasite.

In general, the parasite follows a simple rule: First grow, then divide. This is true for the parasite body, the organelles such as the apicoplast and mitochondrion, and partly also for the nuclei and the parasite membrane, which finally is segregated to the forming merozoites. These different events are now discussed one by one in more detail.

Having rounded up, the trophozoite does not change morphologically for several hours but instead synthesizes DNA. From approximately 20 to 22 hours after infection, nuclear replication begins and the parasite enters schizogony. During schizogony, nuclei are replicated and the parasite grows to a large multinucleated syncytium, the schizont (Figure 2.2C). Nuclear replication is one of the fastest known, with the parasite producing up to 20,000 nuclei in approximately 24 hours in the case of rodent *Plasmodium* species. This is equivalent to one nuclear division every 2 hours. Interestingly, irradiated sporozoites that are used for vaccination approaches develop to the trophozoite stage but do not enter the nuclear replication phase, probably due to nonreversible DNA damage (Meis and Verhave 1988).

To achieve the very fast multiplication rate, the parasite has dramatically altered the cell cycle. Remarkably, it does not bother dissembling the nuclear envelope (Aikawa 1967), which is normally a hallmark of mitosis, and in addition there is initially no immediate fission of nuclei upon DNA replication, with parasites forming a nuclear reticulum (Meis and Verhave 1988). Nuclei have multiple spindles, as is seen in early oocysts (Canning and Sinden 1973; Schrevel 1977). Whereas another apicomplexan parasite, *Sarcocystis neurona*, continues to replicate its genetic material as a nuclear reticulum until shortly before daughter cell formation, forming a 32N polyploid nucleus (Vaishnava 2005), the *Plasmodium* exoerythrocytic parasite leaves the nuclear reticulum phase after two or three rounds of DNA replication and then initiates karyokinesis. In this closed mitosis, the spindles are thought to be embedded within the nuclear envelope, with microtubules then forming within the nucleus (Gerald 2011). It remains unclear how nuclear division is controlled, but the process appears to be asynchronous.

In parallel with nuclear replication, other organelles like the ER, mitochondrion, and apicoplast begin to expand. The apicoplast, not being identified until 1996 (Kohler 1997; McFadden 1996), is not described in EM studies preceding this time. Studies refer frequently to multiple mitochondria (Meis 1985b). These are reported to lie primarily beneath the plasma membrane. We now know that the mitochondrion, despite its considerable growth by 28 to 33 hours after infection, remains as a single organelle (Stanway 2009). Single-slice confocal images, like EM, appear to show multiple mitochondria, but imaging of stacks and three-dimensional reconstructions clearly reveal the mitochondrion to be an extensive branched but single structure (Figure 2.4). It lies close to the plasma membrane, but the mechanisms responsible and the significance of this are unknown. It is hypothesized that a close proximity of the parasite mitochondrion to the plasma membrane and of host cell mitochondria to the PVM might facilitate lipoic acid uptake by the parasite, a process that appears to be necessary for the lipolyation of parasite proteins and hence growth and survival (Deschermeier 2012).

From the early stages of parasite development, the parasite grows extensively, from a diameter of 4 to 10 μm at 21 hours after infection *in vivo* to a diameter of up to 40 μm at 48 hours after infection in the case of rodent *Plasmodium* species. Such an enormous growth in a relatively short time is not trivial. The parasite needs to mobilize nutrients for growth, lipids for membrane expansion (organelles, plasma membrane, PVM), and plenty of nucleotides for the rapidly replicating DNA. For these tasks, the parasite is thought to rely heavily on host cell components, but it still remains to be solved how it accesses them. Possible sources are nutrients in the host cell cytoplasm, the ER, and vesicular components of the secretory system and it has indeed been shown that parasites are closely linked to the host cell ER and to the Golgi apparatus (Figure 2.3).

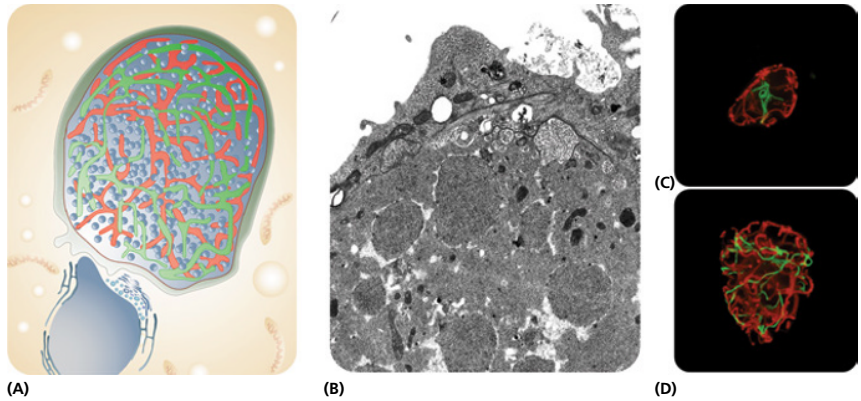

Figure 2.4 Organelle development in *P. berghei* liver schizonts. *A,* Scheme of a schizont with many nuclei *(blue),* one mitochondrion *(red),* and one apicoplast *(green). B,* TEM image showing a cross section of a schizont with mitochondria and apicoplast. *C* and *D,* Live imaging of early schizont *(C)* and late schizont *(D)* expressing a red fluorescent mitochondrion marker protein and a green fluorescent apicoplast marker protein. (*See insert for color representation of this figure*.)

It is still not yet clear how the parasite can make use of these connections. One possible scenario is that host cell vesicles fuse with the PVM, releasing the contents into the PV lumen. Still, the content would consist of macromolecules like glycosylated proteins, which cannot freely cross the parasite membrane. It has been shown that the PV of blood-stage parasites contains plenty of proteases and other degradation enzymes (Nyalwidhe and Lingelbach 2006), and it is reasonable to assume that this is true also for liver-stage parasites. Perhaps some of these enzymes are responsible for degrading macromolecules to their single components like amino acids, glucose, and lipids. Easier access to such small molecules could be provided by lysosomes, which are digestive vacuoles of the cell and thus already contain small molecules that are easier to transport. It has been suggested that lysosomes indeed fuse with the PVM and thus may supply nutrients for the parasite (Lopes da Silva 2012). Another source of nutrients is of course the host cell cytoplasm, and molecules from here need to be imported across the PVM into the parasite. Large pores appear to be formed in the PVM, presumably of parasite origin, and these allow the passage of such molecules, less than 855 Da (Bano 2007). However, uptake of other larger molecules must be via transporters located in the PVM and parasite plasma membrane. Proteins responsible for allowing such transport remain to be identified.

As a result of parasite exploitation of its resources, the host cell is forced into starvation and it is known that amino acid starvation induces autophagy (Yang and Klionsky 2010). Autophagy is a process by which self-digestion of cell components is used to overcome phases of starvation and to provide nutrients for the most essential functions of the cell. Again, the parasite seems to take advantage of this system, but at a high cost. Autophagy is also a method of eliminating intracellular pathogens (Mizushima 2008), and it has indeed been shown that a considerable number of liver-stage parasites are eliminated by autophagy (Prado 2015). The parasites that escape this elimination, however, benefit from the additional supplies of nutrients and can grow to a size that exceeds the volume of a normal hepatocyte (Meis and Verhave 1988).

For the parasite to expand in size, the PVM that surrounds it must also increase in size. Where the additional membrane comes from to allow expansion is so far unclear, but it seems likely that it again may originate at least partly from host cell material. This would not be so surprising because

the PVM is originally derived from host cell plasma membrane, and it is well known that cellular organelles can exchange membranes (Kornmann 2009). A close association has been observed between the PVM and host cell endoplasmic reticulum (Jayabalasingham 2010), which could provide membrane for PVM expansion and might be the reason for the close proximity of the parasite to the host cell nucleus. As the parasite grows, the host cell becomes distended, but its cytoplasmic volume is decreased and this likely coincides with a loss of some host cell organelles, which again could contribute to membrane of the PVM.

While the parasite grows, its organelles also greatly expand. The mitochondrion and apicoplast become enormous branched networks that extend throughout much of the cytoplasm (Figure 2.4) (Stanway 2011). Both organelles appear to remain as single structures. Obviously, continuous growth and branching is faster and more economical for the parasite than the constant fission and segregation processes that are seen in other systems. Obvious connections between the apicoplast and mitochondrion that are seen in the blood stage are not present during exoerythrocytic development. One study, however, provides evidence that at least one metabolite (lipoic acid) is exchanged between the apicoplast and mitochondrion during the liver stage (Falkard 2013). Knockout of the *LipB* gene, which is essential for lipid biosynthesis in the apicoplast, resulted in reduced lipidation of the mitochondrial enzyme BCDH, strongly indicating a link between the two organelles. Perhaps a few contact points between the organelles are enough to permit this limited exchange, allowing the parasite to be less dependent on scavenging lipoic acid from host cell mitochondria.

Prior to the formation of daughter merozoites, the schizont passes through a short-lived but fascinating cytomere stage, characterized by invagination of the parasite membrane to first divide the schizont into the characteristic "islands of cytoplasm" (Meis and Verhave 1988). These invaginations then continue until single merozoites are formed. The origin of merozoite plasma membrane is therefore at least in part the plasma membrane of the schizont (Graewe 2011; Sturm 2009). However, invagination results in a huge increase in the membrane surface, and there is a great demand for newly synthesized membranes. This is illustrated by the fact that *LipB* knockout parasites, which are partially impaired in membrane synthesis, cannot properly complete merozoite formation (Falkard 2013). Others refer to the increased membrane surface as arising from a dilation of the rough endoplasmic reticulum (Meis 1988). It is thought that the cytomere stage might exist to increase the surface area of the plasma membrane and therefore allow the synchronous formation of very large numbers of merozoites.

The cytomere also appears to be the stage where the parasite arranges its organelles ready for segregation into daughter cells. Nuclei align close to the plasma membrane, appearing to be in a ring- or sphere-like arrangement by widefield or confocal microscopy. The mitochondrion appears to be clumped within these rings of nuclei, whereas the apicoplast lies close to nuclei and beneath the plasma membrane. The apicoplast divides and the mitochondrion forms finger-like structures that each point toward the direction of a nucleus (Stanway 2011). The mitochondrion then also divides, with each nucleus being closely paired with a single apicoplast and mitochondrion (mentioned by Aikawa in 1967; the apicoplast was referred to as the spherical body; Aikawa 1967). Further invagination of the plasma membrane leads to the generation of single merozoites, each containing a nucleus, apicoplast, and mitochondrion.

Protein export from the parasite into the host cell

It has already been pointed out that the host cell clearly recognizes the invading parasite and tries to isolate it in a LC3-positive compartment (Prado 2015). Some parasites can obviously evade this host cell defense mechanism, and the question is, why the host cell does not use an alternative

mechanism to eliminate the parasite? Inducing apoptosis would be an appropriate cell response to the invasion of a pathogen, but the parasite also prevents this (Leiriao 2005; van de Sand 2005). It actively blocks host cell apoptosis, and it has been suggested that an inhibitor of cysteine proteases (ICP) has the capacity to inhibit the proteolytic machinery that executes apoptosis (Rennenberg 2010). Although ICPs probably do not inhibit caspases, caspase activation has never been observed in infected hepatocytes (Sturm 2006). Thus, there might be additional levels on which the parasite interferes with the host cell signaling that would normally result in apoptosis. One option is the secretion of proteins across the PM, the PV, and the PVM into the host cell cytoplasm or even then targeted to the host cell nucleus.

Protein export into the host cell is thought to require specific features such as signal sequences and a Pexel (*Plasmodium* export element) motif (Marti 2004), but alternative routes might also exist. In fact, Pexel-containing proteins appear to be very important in *P. falciparum* blood-stage parasites, but very few Pexel-positive proteins have been identified in rodent parasites, and even fewer of them have been shown to be functional. In fact, transport of CSP into the host hepatocyte was originally suggested to be mediated by Pexel motifs found in the N-terminal domain of the protein (Singh 2007), but this Pexel-dependent transport of CSP has been proven wrong (Cockburn 2011). Although the transport route of CSP is not yet clear, it is accepted that it ends up in the host cell cytoplasm, and thus alternative transport mechanisms must exist. Once in the host cell cytoplasm, CSP appears to be transported into the nucleus to inhibit nuclear factor κB (NFκB)-dependent inflammatory immune responses (Singh 2007).

Considering the notion that Pexel-mediated transport motifs are mainly restricted to *P. falciparum* blood-stage parasites, other *Plasmodium* species might differ substantially in terms of protein export. It might even be possible that different transport mechanisms exist in different developmental stages. One possible export route has already been described by Meis and Verhave as a vesicle transport from the cytoplasm to the parasite plasma membrane (Figure 2.5A–C)(Meis and Verhave 1988). Careful ultrastructural analysis of developing liver schizonts revealed a highly interesting phenomenon, which might have strong implications for our current thinking about protein transport from the parasite across the PV and the PVM. Meis and Verhave describe vesicles of different sizes fusing with each other and with the parasite membrane, releasing the "fluffy content" into the PV (Meis and Verhave 1988). However, some of these vesicles that have a similar content and that are thus thought to be of parasite origin are also found in the host cell (Figure 2.5D), where they might release their content by fusion with host cell membranous organelles or by simple rupture. Depending on how these vesicles are originally formed in the parasite, proteins transported in these vesicles might not need a signal peptide to reach the PV. This would be the case if these vesicles are formed like autophagosomes, surrounding portions of cytoplasm. In support of this view, it has been shown that certain parasite proteins can localize in the cytoplasm and in the PV (Rennenberg 2010). On the other hand, if the vesicles described by Meis and Verhave (Meis and Verhave 1988) are derived from the ER or Golgi, one would expect that only proteins with typical signal peptides have access. It is not clear, however, how proteins within these vesicles get access to the PVM and even into the host cell cytoplasm.

It has been shown that the *P. berghei* protein LISP2 is also exported into the host hepatocyte in a Pexel-independent manner (Orito 2012). LISP2 contains a signal sequence, but the export route is not clear. Normally the signal sequence allows a protein to enter the ER. From there it is transported in vesicles to the Golgi and then to its final destination, which might be the plasma membrane but also other membranous compartments in the cell such as endosomes or lysosomes. If LISP2 takes the traditional export route via the ER, the question is how it can cross the PVM to finally enter the host cell. Interestingly, it has been shown that LISP2 is transported in LISP1-positive vesicles to the parasite membrane to be released into the PV. Some vesicles, however, are

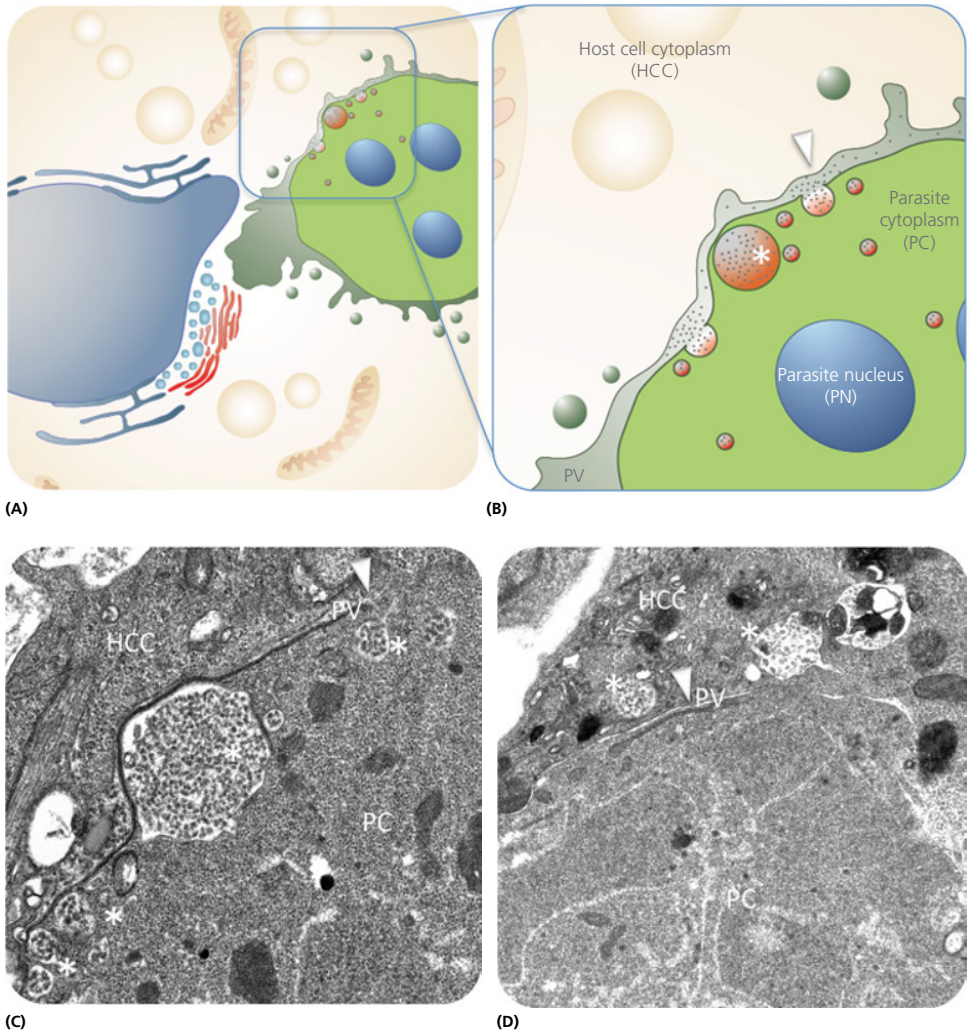

Figure 2.5 Vesicle transport in the parasite and delivery into the host cell cytoplasm. *Upper panels,* The schemes are an overview *(A)* and a magnified area of this overview *(B)* highlighting the vesicle fusion with the parasite plasma membrane and the release of the content into the PV. In the *lower panels,* two TEM images are presented. *C* shows the vesicles accumulating at the plasma membrane. *D* shows the vesicles delivered to the host cell. HCC: host cell cytoplasm; PC: parasite cytoplasm; PN: parasite nucleus, PV: parasitophorous vacuole; asterisks mark parasite-derived vesicles.

even transported across the PVM into the host cell. How this happens is still unclear, but it is tempting to speculate that the vesicles observed by EM studies as appearing to enter the host cell (Meis and Verhave 1988) (Figure 2.5C,D) contain LISP2. Upon release from the vesicles, LISP2 is found in the cytoplasm and the nucleus of the host cell (Orito 2012), suggesting a function in gene regulation. Whether LISP2 regulates signal transduction pathways is not clear, but one can assume that the described transport is not restricted to LISP2, and many other parasite proteins could be transported in this manner, perhaps also including CSP since the Pexel-dependent transport of CSP has been excluded (Cockburn 2011).

Parasite egress

In vivo exoerythrocytic *P. berghei* development results in the generation of thousands of merozoites within a single hepatocyte. How do hepatocyte-derived merozoites, which are not known for their capacity to migrate over longer distances or to transmigrate cells, enter the blood vessel to infect red blood cells? A first hint came again from Meis and Verhave in their famous review where they present several EM images showing that a "mass of merozoites, kept together by stroma, bulges out into the sinusoid and groups of merozoites are released" (Meis and Verhave 1988). This initial observation was extended by the investigations of Sturm and coworkers, who not only confirmed the existence of the aforedescribed phenomenon but could show that the masses of merozoites are still surrounded by a host cell membrane (Graewe 2011; Sturm 2006). They therefore suggested naming these merozoite-filled vesicles merosomes, which is the term now commonly used in the scientific community (Figure 2.2D and Figure 2.6).

It is now clear that merosomes form after the parasite-induced collapse of the PVM and the subsequent death of the host cell. Again, the fact that the PVM ruptures before the disintegration of the host cell membrane was already well known in the 1980s, and merosomes had already been observed *in vitro* (Hollingdale, personal communication) long before this phenomenon was finally

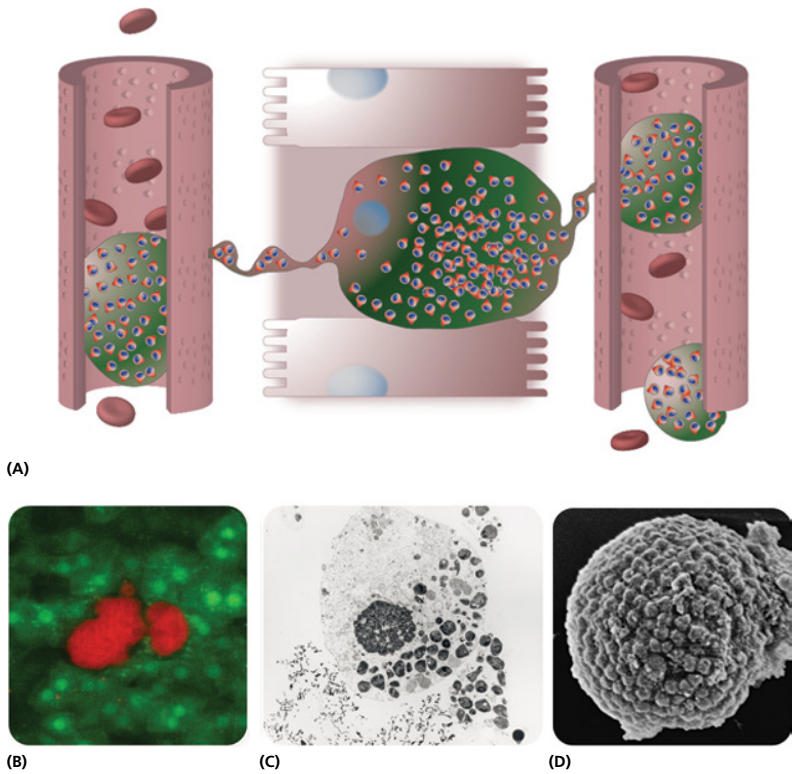

Figure 2.6 Merosome formation *in vivo* and *in vitro:* The scheme depicts the events surrounding merosome formation *in vivo. A,* Upon PVM rupture, the host cell detaches from the neighboring cells; vesicles (merosomes) bud off and are constantly filled with infectious merozoites. *B,* Intravital image of mCherry-expressing parasites infecting an LC3-GFP-expressing transgenic mouse. *C,* TEM of a detached cell with a budding merosome. *D,* REM image of a merosome filled with many mature merozoites. (Image from Heussler, 2012.) (*See insert for color representation of this figure.*)

published. Together, the original ultrastructural evidence and the more recent molecular dissection of merosome formation has closed a long-standing gap in the life cycle of *Plasmodium* parasites, namely how the hepatocyte-derived merozoites reach the blood stream to infect red blood cells. It is somehow ironic that the first description of merosomes came from the Bernhard Nocht Institute for Tropical Medicine in Hamburg, where more than 100 years ago Schaudinn argued against any pre-erythrocytic development of *Plasmodium* parasites (Schaudinn 1903; Sturm 2006). The existence of merosomes also now been proven in the human parasite *P. falciparum* (Vaughan 2012), confirming that rodent *Plasmodium* species represent good models for studying parasite egress from hepatocytes.

Now that the existence of merosomes and their transport across endothelia to reach blood vessels is confirmed, analysis of the molecular details of merozoite liberation from infected hepatocytes has been the focus. Already during merozoite formation, parasite proteins begin to leak into the host cell, thus demonstrating that the PVM becomes increasingly permeable before being completely disrupted (Rennenberg 2010; Schmidt-Christensen 2008; Sturm 2006). Live imaging of a parasite strain expressing a fluorescent PVM marker protein confirmed that the PVM breaks down toward the end of the liver stage while the host cell membrane stays intact (Graewe 2011). The same study revealed that breakdown of the PVM takes place within a relatively short time frame and therefore must be a highly efficient process. The first class of enzymes one would consider to act on membranes are lipases but it remains to be shown whether the parasite secretes or activates lipases to destroy the PVM.

It is known, however, that proteases can also destabilize membranes by removing integral membrane proteins. Indeed, PVM breakdown can be inhibited by E64, an inhibitor of cysteine proteases, which indicates a role for this class of proteases, directly or indirectly in PVM breakdown (Sturm 2006). Cysteine proteases appear to also mediate parasite liberation from erythrocytes, and there are many features common to the liberation of merozoites from both cell types (Blackman 2008). Despite the similar action of proteases during *Plasmodium* egress from hepatocytes and erythrocytes, there are also clear differences, most likely because of the different nature of the respective host cells and the different needs of the parasite. Upon breakdown of the PVM, exoerythrocytic merozoites stay for an extended period of time in hepatocyte-derived vesicles until they reach their final destination, where they can safely infect erythrocytes (Sturm 2006). In contrast, egress from erythrocytes needs less coordination because liberated merozoites can infect a new erythrocyte within seconds (Gruring 2011).

After invasion of erythrocytes, *P. falciparum* activates nonselective cation channels in the erythrocyte membrane, presumably enabling easy access to sodium and calcium (Kasinathan 2007). Usually, the activation of these channels leads to a rise in intracellular calcium levels and triggers eryptosis, the programmed cell death of erythrocytes. Characteristic features of eryptosis, such as the activation of calpain, do not occur until the late blood stage. In *Plasmodium* infections, this process is delayed, possibly due to uptake of calcium by the parasite (Kasinathan 2007). Even then these features seem to be tailored to support parasite development, because it has been shown that in the absence of host cell calpain-I, *P. falciparum* is incapable of egressing from the erythrocyte (Chandramohanadas 2009). It has been proposed that host cell calpain-I also becomes activated in the liver stage, but since *Pb*ICP appears to inhibit calpain-I (Pandey 2006) and both are found in the host cell upon PVM breakdown, calpain-I is not likely to play a major role in parasite egress from hepatocytes. Additionally, the increased uptake of calcium by exoerythrocytic merozoites (Sturm 2006) would further argue against activation of calpain-I. It has even been demonstrated that calcium uptake by exoerythrocytic merozoites blocks the switch of phosphatidylserine residues from the inner leaflet to the outer leaflet of the membrane, allowing the parasite to interfere with host cell signaling and to avoid attack by phagocytes (Graewe 2011; Heussler 2010; Sturm 2006).

It has been suggested that the protein LISP1 is involved in PVM disruption (Ishino 2009). LISP1 localizes to the PVM, and deletion of the *lisp1* gene results in an inability of the parasite to escape from the PV. LISP1 itself, however, has no recognizable functional protease domain and is therefore suspected to be either a membrane receptor for proteases or to be involved in the processing of proteases for activation (Ishino 2009).

A more likely scenario is that the protease responsible for PVM breakdown is expressed by the parasite itself. It has been shown that late liver stage *P. berghei* parasites express a number of putative serine repeat antigen (SERA) proteases, which become processed briefly before merozoite release (Putrianti 2010; Schmidt-Christensen 2008). SERAs are target proteins of a protease cascade involving DPAP3 (dipeptidyl-aminopeptidase 3) and subtilisin I activation and can therefore serve as a good readout for the activation of such a protease cascade (Blackman 2008). There is so far no evidence that SERAs are indeed proteases, but even if they do not act as proteases themselves, their processing strongly suggests a functional proteolytic cascade. Importantly, both proteases, DPAP3 and subtilisin, are expressed by late liver stage parasites, further supporting the idea of a functional proteolytic cascade that induces PVM rupture.

Even more interesting is whether one of the proteases in this cascade is responsible for triggering host cell death that is induced upon PVM breakdown (Heussler 2010; Sturm 2006). This parasite-dependent host cell death is characterized by cytochrome c release from mitochondria and nuclear condensation but with retention of an intact cell membrane (Graewe 2011; Heussler 2010; Sturm 2006). It therefore clearly differs from necrosis, which typically includes the swelling and rupture of the cell. It is also not an unorganized cell death, which could be induced if pathogen-containing vacuoles rupture to release destructive content into the host cell cytoplasm. We can exclude this because when premature rupture of the PVM was induced, releasing parasite material into the cytoplasm, the host cell stayed intact (Nagel 2013). Parasite-induced host cell death appears to be an ordered form of cell death and, at a first glance, resembles apoptosis. However, it differs in many respects and appears to be a unique *Plasmodium*-induced cell death mechanism (Heussler 2010).

Whether apoptosis is triggered or not is usually decided by the balance between pro- and anti-apoptotic stimuli. Both the integration process and the initiation of apoptosis often take place in the mitochondrion. Our observation that host cell mitochondria disintegrate rapidly after PVM breakdown (Graewe 2011) could be considered a typical apoptotic feature. Upon closer examination, however, we found that parasite-dependent host cell death lacks important hallmarks of apoptosis such as DNA fragmentation, caspase cascade activation, and loss of phosphatidylserine asymmetry (Graewe 2011; Heussler 2010; Sturm 2006). During its growth phase, the parasite actively blocks host cell apoptosis, and this block might still exist once the PVM ruptures.

A possible candidate for this inhibition is the previously mentioned cysteine protease inhibitor PbICP, which is not only present in the host cell cytosol early after invasion but also floods the host cell upon PVM disruption (Rennenberg 2010). It has been shown to inhibit apoptosis when expressed in CHO cells. Because it is effective against cathepsin L, but not against other cathepsin proteases like B- and C-type cathepsins, PbICP could in theory block host cell effector proteases while allowing some parasite proteases, of for example the B- or C-type, to remain functional. Possible candidates are all serine proteases, such as subtilisin I and the cysteine protease DPAP3, because DPAP3 shares some features of cathepsin B-like proteases. However, it is more likely to be a protease that is not expressed in the blood stage because there PVM and host cell membrane rupture are almost simultaneous events (Blackman 2008; Gruring 2011). Because the plasma membrane of infected hepatocytes does not break down immediately upon PVM disruption, the protease that destroys the erythrocyte membrane might therefore either not be synthesized in the liver stage or might indeed be inhibited by specific parasite factors like the aforementioned inhibitor PbICP (Rennenberg 2010).

Taken together, toward the end of the *Plasmodium* liver stage, the host cell undergoes an unusual programmed cell death. It is not yet fully understood how this process occurs, but specialized parasite proteases and protease inhibitors might both play a role. The result is a host cell that detaches from its surroundings and contains free exoerythrocytic merozoites in its cytosol. The next important step is then the formation of merosomes that bulge into the blood vessel, where they separate from the mother cell to travel from the liver, through the heart, and into lung capillaries where merozoites are finally released (Baer 2007).

The role of innate immune responses during merosome formation

In his EM studies, Meis already noted that late liver stage parasites are penetrated by neutrophils and monocytes (Meis 1987; Meis and Verhave 1988). He speculated that rupture of the infected hepatocyte might attract phagocytes and pointed out that innate immune responses against the tissue stage of the parasite might play an important role. Later these phagocyte infiltrations to the site of infection have been confirmed (Epiphanio 2008; Khan and Vanderberg 1991; van de Sand 2005). Despite this, the most important question remained: what attracts the phagocytes to the infected hepatocyte during late liver stages? It has been suggested that the observed infiltration is due to damage of cells transmigrated by *Plasmodium* sporozoites (Frevert 2004). However, this does not explain why some infected hepatocytes are surrounded by phagocytes and others not, and the timing of the different events argues against it. Neutrophil recruitment is a fast event, and as they constantly patrol the liver, a delay of two days in neutrophil infiltration (from sporozoite transmigration to merozoite development) is very unlikely. A better explanation is that phagocytes are attracted when host cell death is induced during merozoite egress (van de Sand 2005). Dying cells secrete "find me" signals (nucleotides, small lipids, CX3CL1) to recruit phagocytes for their removal (Ravichandran 2011), but this remains to be shown for *Plasmodium*-infected cells.

Nevertheless, host cell death is a rather asynchronous event and fits very well with the observed pattern of phagocyte infiltration. However, it still remains to be shown whether phagocytes infiltrate before or after merosome formation. This is a very important question because it is a plausible scenario that phagocytes attracted by the dying cell in entering the liver tissue from blood vessels pave the way for merosome migration in the opposite direction, namely into the blood vessel. This would nicely explain how even a dying cell can still support merozoite transport into the blood vessel. But why would the attracted immune cells not phagocytose the dying cell and merosomes?

A hallmark of dying cells is the release of Ca^{2+} from internal stores and the subsequent switch of phosphatidylserine (PS) residues from the inner leaflet to the outer leaflet of the membrane. This PS switch is one of the "eat me" signals for phagocytes (Ravichandran 2011). However, detached cells and merosomes containing viable merozoites are not recognized as dead cells, although Ca^{2+} is released from the internal stores. Thousands of merozoites take up the released Ca^{2+} (Graewe 2011; Heussler 2010; van de Sand 2005), and in this way the outer membrane of the merosome is kept clear of PS residues. This might be one reason the liver stage produces so many merozoites. Fewer might not be enough to control intracellular Ca^{2+} levels, allowing a PS switch to occur.

Another reason for the large number of merozoites produced might be that merosome formation is a relatively inefficient process and needs large numbers of parasites to successfully deliver at least some to the blood vessel. Once merosome formation is finished, the residual parasites inside the mother cell become apoptotic and are finally phagocytosed (van de Sand 2005). An important question is whether phagocytosis triggers adaptive immune responses of the host. This is not very likely because the liver is an immune-privileged organ, suppressing induction of immune responses.

Because it has been shown that adaptive cell-mediated immune responses against tissue forms in the liver exist, however, the priming must come from the periphery, and this has indeed been shown to be the case (Overstreet 2008; Sinnis and Zavala 2012). Adaptive immune responses against the liver stage of the parasite are topics of intense research because many vaccine approaches are based on this kind of immune response, but it is beyond the scope of this chapter to go into detail. The interested reader is referred to recent reviews and other chapters in this book.

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CHAPTER 3

Molecular basis of erythrocyte invasion by *Plasmodium* merozoites

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Malaria is one of the three major global infectious diseases that annually accounts for around 1.2 million deaths, most of which occur in young children and pregnant women residing in some of the poorest countries of Africa (Snow, 2005; Murray, 2012). Although there are five *Plasmodium* species that can infect humans, most cases of human malaria are caused by the infection of *Plasmodium falciparum* or *Plasmodium vivax*. Recently, *Plasmodium knowlesi*, which was earlier believed to infect only monkeys (simian malaria) has been confirmed to cause the fifth human malaria (Singh, 2004; Kantele, 2011). *P. falciparum* causes the most severe form of the disease, cerebral malaria, which is responsible for most of the deaths attributed to malaria. Due to fundamental differences in the biology of *P. vivax* that are enumerated later, *P. vivax* malaria was believed to be benign compared to *P. falciparum* malaria and has been relatively neglected (Galinski and Barnwell, 2008). However, it is now apparent that *P. vivax* malaria is geographically more widespread across the world than *P. falciparum* malaria and can also cause severe disease conditions as well as deaths (Galinski and Barnwell, 2008). This chapter focuses primarily on the erythrocyte-invasive properties of the two human malaria parasites, *P. falciparum* and *P. vivax*.

As eloquently described in the previous chapters, *P. falciparum* and *P. vivax* have a complex life cycle involving both the vertebrate human host and the female *Anopheles* mosquito as the transmitting vector and invertebrate host. The parasite life cycle begins with the bite of the infected female *Anopheles* mosquito, when she is pregnant, and the injection of sporozoites during a blood meal. The sporozoites home to the liver, where they grow and multiply several thousand-fold within hepatocytes to form exoerythrocytic schizonts. These exoerythrocytic schizonts release invasive forms of the parasite into the blood stream that are known as merozoites, which invade human erythrocytes to initiate the asexual blood-stage of the parasite's life cycle. Within the infected erythrocyte, the parasite grows over a period of 48 hours through the ring, trophozoite, and schizont stages (Figure 3.1). During this period of erythrocytic schizogony, the parasite multiplies to form 16 to 32 daughter merozoites that are released upon egress and subsequently infect other uninfected naïve human erythrocytes (Figure 3.1). This asexual erythrocytic phase of the *Plasmodium* life cycle accounts for the clinical symptoms and pathology associated with the malarial disease.

Thus, *Plasmodium* is primarily an obligate intracellular organism that remains in an extracellular motile form for very short periods of time. In order for the parasite to complete its life cycle, it is critical that the different invasive stages (sporozoite, merozoite, ookinete) that are extracellular recognize and invade the appropriate host cell types. In this regard, the ability of *Plasmodium*

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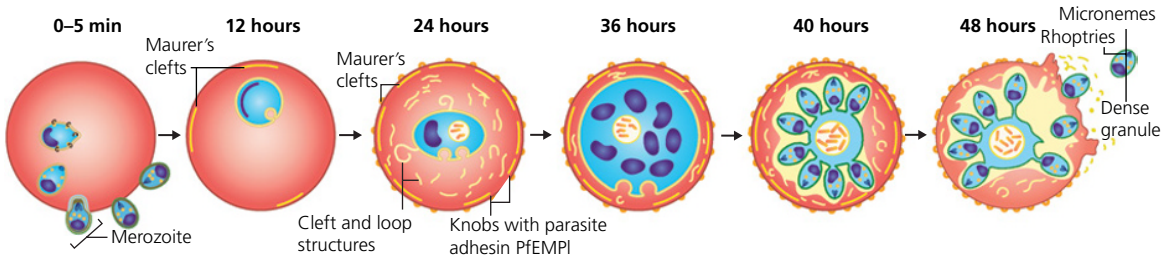


Figure 3.1 Asexual blood-stage life cycle of *Plasmodium*. Different stages of *P. falciparum* development in the erythrocyte are depicted. The merozoites attach the erythrocyte, apically reorient, form the junction, and invade erythrocytes. Within the infected erythrocyte, the parasite is present in the parasitophorous vacuole (PV), where it develops through the ring (0–24 hours), trophozoite (24–36 hours), and schizont stages (40–48 hours). During maturation, the erythrocyte surface gets remodeled with the formation of membrane-bound structures (cleft and loop structures, Maurer's clefts) in the erythrocyte cytoplasm and knobs on the erythrocyte membrane displaying the surface variant protein PfEMP1 (*P. falciparum* erythrocyte membrane protein 1). At around 48 hours, the infected erythrocyte undergoes egress, and 16 to 32 daughter merozoites are released into the blood stream, where they further invade nascent erythrocytes. *Source:* Maier AG, Cooke BM, Cowman AF, Tilley L. 2009. Malaria parasite proteins that remodel the host erythrocyte. *Nature Reviews Microbiology.* 7:341–354.

parasites to invade erythrocytes can be considered a significant virulence factor because the buildup of high parasite densities in the host blood stream leads to severe pathology and morbidity.

Invasion of erythrocytes by *Plasmodium* merozoites is an intricate biological process that is interwoven between different physiological steps and requires a cascade of specific ligand–receptor interactions at the parasite–erythrocyte interface. These numerous molecular interactions enable *Plasmodium* merozoites to invade erythrocytes through a number of alternative pathways. This redundancy was earlier considered a hallmark of *P. falciparum,* but now it is apparent that *P. vivax* has also evolved a large molecular machinery to invade human erythrocytes.

The 23 Mb *Plasmodium* genome comprises 5300 genes, of which 2700 are predicted to be expressed during the asexual blood-stage (Gardner, 2002; Bozdech, 2003; Le Roch, 2003). Extensive research over several decades on the ligand–receptor interactions that mediate erythrocyte invasion has improved our understanding of this complex process, there still remain a number of unanswered questions regarding the molecular basis of erythrocyte invasion. A large number of hypothetical genes that are expressed in merozoites have not yet been characterized and encode proteins whose function remains unknown. Thus, the complete repertoire of parasite molecules that play a role in the invasion process is not known. Further, of those parasite proteins known to be involved in erythrocyte invasion, the precise function for many remains undefined (Table 3.1). Hence, a complete understanding of the molecular and cellular biology that underlies the erythrocyte invasion process is absolutely critical not only as a basic research exercise to enhance our knowledge but also for the development of successful therapeutic and prophylactic strategies such as drugs and vaccines against malaria parasites.

Nevertheless, advances in several new technologies based in genomics, proteomics, microarrays, genetic manipulation and methods to produce invasive extracellular merozoites have significantly enhanced our understanding of the complex invasion process at the molecular level. A number of articles have reviewed our understanding of erythrocyte invasion (Gaur, 2004; Cowman and Crabb, 2006; Iyer, 2007; Gaur and Chitnis, 2011; Tham, 2012; Cowman, 2012). In the present chapter, we have tried to build on these articles and have reviewed the current state of our knowledge about the molecular basis of erythrocyte invasion by *P. falciparum* and *P. vivax*.

Table 3.1 Characteristic features of Invasion related proteins of *P. falciparum*.

Name	Accession Number	Localization	Erythrocyte Binding	Receptor	Genetic Disruption	Protein Function & Characteristics
Merozoite Surface Proteins (MSPs)						
MSP-1	PF3D7_0930300	Surface	Yes	Band 3	NS	GPI-linked; Involved in Erythrocyte Attachment; MSP-1/MSP-9 Co-complex binds Band 3
MSP-2	PF3D7_0206800	Surface	Not Known		NS	GPI-linked; Constitutes fibrillar merozoite coat; Potent target of Antibody dependent cellular inhibition
MSP-3	PF3D7_1035400	Surface	Not Known		S	Soluble; Constitutes fibrillar merozoite coat; Potent target of Antibody dependent cellular inhibition
MSP-4	PF3D7_0207000	Surface	Not Known		S	GPI-linked surface protein with a single EGF domain
MSP-5	PF3D7_0207000	Surface	Not Known		NS	Carboxy terminal single EGF domain
MSP-6	PF3D7_1035500	Surface	Not Known		S	Constitutes the MSP-1/6/7 complex on the merozoite surface; facilitates MSP-DBL erythrocyte binding
MSP-7	PF3D7_1335100	Surface	Yes	P-Selectin	S	Constitutes the MSP-1/6/7 complex on the merozoite surface; facilitates MSP-DBL erythrocyte binding
MSP7-like (MSRP2)	PF3D7_1334800	Surface	Not Known		ND	Putative MSP1 binding protein, translocated to parasitophorous vacuole
MSP7-like (MSRP1)	PF3D7_133500	Surface	Not Known		ND	Putative MSP1 binding protein associated with detergent-resistant membranes
MSP-8	PF3D7_0502400	Plasma Membrane	Not Known		S	Predominantly expressed at ring stage; Processed fragment localized in food vacuole at schizont stage
MSP-9 (ABRA)	PF3D7_1228600	Surface	Yes	Band 3	S	Erythrocyte Attachment; MSP-1/MSP-9 Co-complex binds Band 3
MSP-10	PF3D7_0620400	Surface	Not Known		NS	GPI-linked surface protein; C-terminal double EGF module

(*Continued*)

Table 3.1 (Continued)

Name	Accession Number	Localization	Erythrocyte Binding	Receptor	Genetic Disruption	Protein Function & Characteristics
H101	PF3D7_1035600	Surface	Not Known		S	MSP3-like protein on chr 10; function unknown
H103	PF3D7_1035900	Surface	Not Known		S	MSP3-like protein on chr 10; function unknown
MSPDBL-1	PF3D7_1036300	Surface	Yes	Not Known	S	Erythrocyte Attachment, Interact with MSP-1, Contain DBL domain
MSPDBL-2	PF3D7_1035700	Surface	Yes	Not Known	S	Erythrocyte Attachment, Interact with MSP-1, Contain DBL domain
GLURP	PF3D7_1035300	Surface	Not Known		S	Parasitophorous vacuole protein
Proteins involved in the Junction						
AMA-1	PF3D7_1133400	Microneme	No		NS	Component of the moving Junction; Binds to RON2 protein inserted in the erythrocyte membrane
RON2	PF3D7_1452000	Rhoptry Neck	No		ND	Component of the moving Junction; Inserted in to the erythrocyte membrane and served as AMA-1 receptor
RON3	PF3D7_1252100	Rhoptry Neck	Yes		ND	Erythrocyte binding function demonstrated for recombinant protein
RON4	PF3D7_1116000	Rhoptry Neck	No		ND	Component of the moving Junction; Inserted in to the erythrocyte cytosol and remains associated with RON2 as a complex
RON5	PF3D7_0817700	Rhoptry Neck	No		ND	Component of the moving Junction; Inserted in to the erythrocyte cytosol and remains associated with RON2 as a complex
Thrombospondin domain containing proteins						
MTRAP	PF3D7_1028700	Microneme	Yes	Semaphorin A	NS	TSR domain containing protein associated with the actin-myosin invasion motor
PTRAMP	PF3D7_1218000	Rhoptry bulb	Yes	Not Known	ND	TSR domain containing protein associated with the actin-myosin invasion motor

Erythrocyte Binding Antigens (EBAs/DBLs)						
EBA-140/BAEBL	PF3D7_1301600	Microneme	Yes	Glycophorin C	S	Mediates invasion through the sialic acid dependent pathway
EBA-175	PF3D7_0731500	Microneme	Yes	Glycophorin A	S	Mediates invasion through the sialic acid dependent pathway
EBA-181/JESEBL	PF3D7_0102500	Microneme	Yes	Band 4.1	S	Mediates invasion through the sialic acid dependent pathway
EBL1	PF3D7_1371600	ND	Yes	Glycophorin B	ND	Exist as a pseudogene in many P. falciparum isolates
Reticulocyte binding-like Homologous (PfRH) Proteins						
RH1	PF3D7_0402300	Rhoptry Neck	Yes	Not Known	S	Erythrocyte Binding; Mediates invasion through the sialic acid dependent pathway
RH2a	PF3D7_1335400	Rhoptry Neck	Yes	Not Known	S	Erythrocyte Binding; Mediates invasion through the sialic acid dependent pathway
RH2b	PF3D7_1335300	Rhoptry Neck	Yes	Not Known	S	Erythrocyte Binding; Mediates invasion through the sialic acid independent pathway
RH4	PF3D7_0424200	Microneme	Yes	CR1	S	Binds Complement Receptor 1 (CR1); Mediates invasion through the sialic acid independent pathway
RH5	PF3D7_0424100	Rhoptry bulb	Yes	Basigin (CD147)	NS	Mediates an essential core step in invasion critical for all P. falciparum strains
Other Micronemal Proteins						
Ripr	PF3D7_0323400	Microneme	No		NS	Component of PfRH5-Ripr-CyRPA complex. Contain EGF Like domain
CyRPA (RRMAP)	PF3D7_0423800	Microneme	No		NS	GPI linked membrane anchoring protein of the PfRH5-Ripr-CyRPA complex
PfMA	PF3D7_0316000	Microneme	Yes	Not Known	ND	Erythrocyte Binding Adhesin
GAMA	PF3D7_0828800	Microneme	Yes	Not Known	ND	Erythrocyte Binding Adhesin

(*Continued*)

Table 3.1 (Continued)

Name	Accession Number	Localization	Erythrocyte Binding	Receptor	Genetic Disruption	Protein Function & Characteristics
Other Rhoptry Proteins						
AARP	PF3D7_0423400	Rhoptry Neck	Yes	Not Known	ND	Erythrocyte Binding; Mediates invasion through the sialic acid dependent pathway
RhopH1(CLAG2)	PF3D7_0220800	Rhoptry Bulb	Not Known		ND	Constitutes a high molecular mass rhoptry complex that binds erythrocytes
RhopH1(CLAG3.1)	PF3D7_0302500	Rhoptry Bulb	Not Known		ND	Constitutes a high molecular mass rhoptry complex that binds erythrocytes
RhopH1(CLAG3.2)	PF3D7_0302200	Rhoptry Bulb	Not Known		ND	Constitutes a high molecular mass rhoptry complex that binds erythrocytes
RhopH1(CLAG9)	PF3D7_0935800	Rhoptry Bulb	Not Known		S	Constitutes a high molecular mass rhoptry complex that binds erythrocytes
RhopH2	PF3D7_0929400	Rhoptry Bulb	Not Known		ND	Constitutes a high molecular mass rhoptry complex that binds erythrocytes
RhopH3	PF3D7_0905400	Rhoptry Bulb	Not Known		NS	Constitutes a high molecular mass rhoptry complex that binds erythrocytes
RALP-1	PF3D7_0722200	Rhoptry Neck	Yes	Not known	NS	Putative erythrocyte binding propoerty
ASP	PF3D7_0405900	Rhoptry Neck	Not Known		ND	Putative GPI-anchor; Function unknown
6-Cysteine Proteins						
Pf12	PF3D7_0612700	Apical/Surface	Not Known		S	Member of 6-cys family; surface-only/6-cys domains modeled as similar to T. gondii surface protein SAG1
Pf38	PF3D7_0508000	Surface	Not Known		S	Member of 6-cys family; surface and apical appearance
Pf92	PF3D7_1364100	Surface	Not Known		S	Cysteine-rich surface protein
Pf113	PF3D7_1420700	Surface	Not Known		NS	Putative surface protein
Pf41	PF3D7_0404900	Apical/Surface	Not Known		S	Member of 6-cys family; apical end of merozoites; Forms heterodimer with P12

Abbreviations: S = Knockout Successful; NS = Knockout Not Successful; ND = Knockout Not Done.

The structure of the merozoite

Plasmodium belongs to the family of Apicomplexans, which as their name suggests are parasites that have an apical structure and possess characteristic apical organelles near the tip of the apex. Apart from the *Plasmodium* species, the phylum Apicomplexa comprises a large number of parasites that infect humans and animals such as *Eimeria, Babesia, Theileria, Toxoplasma,* and *Cryptosporidium*. Unlike *Plasmodium*, three members—*Toxoplasma*, *Eimeria* and *Cryptosoridium*—have life cycles that transmit between vertebrate hosts itself. The name of the taxon *Apicomplexa* is derived from two Latin words—*apex* (top) and *complexus* (infolds)—and refers to a set of specialized organelles (micronemes, rhoptries) at the apical end that are known to harbor and secrete proteins involved in host cell invasion (Figure 3.2).

The invasive forms of the parasite are called *zoites* (*Plasmodium:* sporozoite, merozoite; *Toxoplasma:* tachyzoite), which are elongated and polarized, with an apical morphology that determines the direction of movement. The *Plasmodium* blood-stage merozoite is the smallest of all parasite stages in the life cycle (Figure 3.2). It is ovoid, being broad at the posterior end and gradually tapering toward the anterior apical end. It is probably one of the smallest protists known, with dimensions of 1.5 to 2.5 μm long and 1.0 to 2.0 μm wide, that has evolved to invade and reside in again one of the smallest vertebrate cells, the human erythrocyte (Figure 3.2). The size of the merozoite varies between *Plasmodium* species: *P. vivax* merozoites are reported to be larger than *P. falciparum* merozoites.

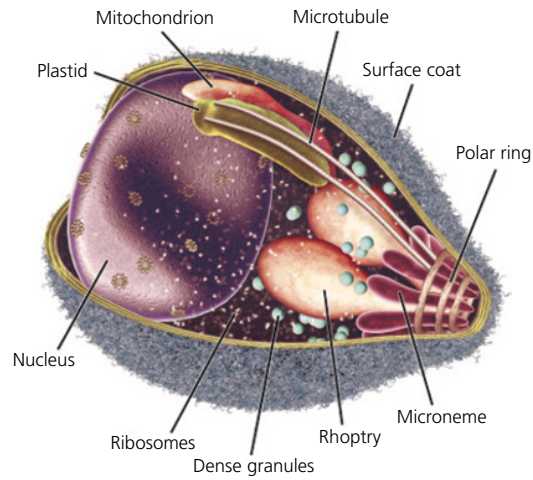


Figure 3.2 Three-dimensional structure of the *P. falciparum* merozoite depicting its architecture and subcellular organelles. The merozoite is a pear-shaped structure with an apical pole that has underlying polar rings. It possesses an outer surface coat that is fibrillar in nature and comprises a number of merozoite surface proteins. Internally, it is characterized by the presence of the apical organelles (rhoptry, microneme) that are a key feature of the apicomplexan organisms and are known to harbor most of the parasite proteins involved in host cell invasion (e.g., EBA, RH, AMA-1). Dense granules are released immediately after invasion and harbor components of the protein translocation machinery that is inserted into the parasitophorous vacuole membrane. The merozoite possesses all the other subcellular organelles characteristically found in eukaryotes, such as the nucleus, mitochondria, endoplasmic reticulum, Golgi, microtubules, and ribosomes. The key structures that are unique to this eukaryotic pathogen are the presence of the apicoplast (a relict of the chloroplast), the subpellicular microtubules, and the inner membrane complex. *Source:* Cowman AF, Crabb BS. 2006. Invasion of red blood cells by malaria parasites. *Cell.* 124(4):755–766. Review.

Merozoites have an outer plasma membrane that encapsulates the regular and vital gamut of organelles including a nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, and ribosomes that are essential for eukaryotic physiology and the characteristic apical organelles: the rhoptries, micronemes, and dense granules. Like all zoites, merozoites have a typical apical prominence that structurally comprises three polar rings, which are made of a cytoskeletal matrix. The polar rings are bound by a triple membranous structure, the pellicle that consists of the plasma membrane and two other membranes that form a network of flattened vesicles underlying the plasma membrane and known as the inner membrane complex. The merozoites possess two or three subpellicular microtubules that are attached to the polar rings and extend on only one side of the merozoite to around two thirds of its length (Figure 3.2).

Over the merozoite plasma membrane lies a structured surface coat, which has been described in the literature as being fibrillar and amorphous. Lawrence Bannister has extensively described the merozoite surface coat in great detail for *P. knowlesi*, and he has reported that the surface constitutes fibrillar clusters that are interspersed between amorphous proteins (Bannister, 1986). The clusters of tufted fibrils are clearly spaced and comprise 5 to 10 filaments (2–3 nm thickness, 18–22 nm length). This fibrillar coat was found to surround the entire merozoites, including its apical pole. Similar structures have also been described for *P. falciparum* (Langreth, 1978) and *P. vivax* merozoites (Galinski and Barnwell, 1996). The merozoite has at least 10 proteins that are known to be associated with its surface and are called merozoite surface proteins (MSPs). Some of the MSPs exhibit the property of forming amyloid fibrils that contribute to the natural appearance of the outer coat of the merozoite and will be discussed in greater detail later.

The apical organelles play an important role in merozoite invasion of the host erythrocyte and harbor a number of parasite proteins that are secreted on the merozoite surface during the different steps of invasion. The rhoptries are two large pear-shaped structures that were the first apical organelles to be discovered by electron microscopy. The name, *rhoptry,* originates from the Greek word *rhoptos,* which means "club," referring to their shape. The pair of rhoptries is attached through a split duct that connects with the apical pole. Micronemes refer to around 40 small and elongated vesicles that localize around the rhoptry duct. The name, *microneme,* originates from the Greek word *neme,* which means "thread-like structure" and refers to their small (micro) size and elongated (thread-like) form. Micronemes have been reported to originate as vesicles from the Golgi apparatus and translocate along the microtubules to the apical pole. For a long time there was a debate whether proteins from the micronemes or rhoptries are secreted first during merozoite invasion. However, a number of reports both on *Toxoplasma* and *Plasmodium* have shown that in fact, micronemal release precedes rhoptry release and that the latter is dependent on the attachment of micronemal proteins with the host cell surface (Carruthers, 1999; Carruthers and Sibley, 1999; Wetzel, 2004; Singh, 2010; Gaur and Chitnis, 2011; Sharma and Chitnis, 2013). This is discussed later in the section on signaling events during erythrocyte invasion.

The third set of apical organelles are the rounded dense granules that are also referred to as microspheres in earlier reports. Dense granules contain proteins such as the ring-infected erythrocyte surface antigen (RESA) that is secreted into the parasitophorous vacuole following invasion (Culvenor, 1991). However, Blackman and colleagues have shown that the dense granules are not all homogeneous, as previously believed, and that their protein composition as well as function could be distinct, leading to the identification of a new set of organelles known as the *exonemes* (Janse and Waters, 2007; Yeoh, 2007). The exonemes have been shown to harbor the serine protease subtilisin-1 (PfSub1), which is an essential protease that is secreted in mature schizonts to trigger egress (Yeoh, 2007). These exonemes appear similar to the dense granules and share their electron-dense characteristic. However, they are fewer in comparison and are larger and elongated compared to the spheroidal RESA contained in dense granules. Such heterogeneity has also been

reported for micronemes, which are known to localize both the AMA-1 and EBA-175 parasite proteins. Mitchell and colleagues have reported that during schizont structural maturation, PfAMA-1 appears in micronemes before EBA-175, implying distinct sets of micronemes harboring different proteins (Margos, 2004).

Similarly, a new organelle in the merozoite has been reported that contains the protease *P. falciparum* rhomboid-1 (PfROM1), which cleaves the transmembrane regions of different invasion-related ligand proteins (Singh, 2007). By immunoconfocal microscopy, PfROM1 was localized to a single thread-like structure, mononeme, on one side of the merozoites proximal to the subpellicular microtubules (Singh, 2007). PfROM1 was not found to be colocalized with the micronemes, rhoptries, or dense granules (Singh, 2007). The identification of the exonemes and mononemes suggests that further comprehensive analysis of the merozoite structure could provide more details on its cellular organization.

The steps of erythrocyte invasion

The process of erythrocyte invasion by *Plasmodium* merozoites is highly complicated and comprises multiple steps, which appears to be similar among all *Plasmodium* species (Figure 3.3). In the first step, the merozoite attaches reversibly to the surface of the erythrocyte followed by apical reorientation, formation of an irreversible tight junction and finally its entry into the parasitophorous vacuole. The entire invasion process is mediated by a number of specific ligand-receptor interactions at the parasite-host cell interface (Table 3.1). *P. falciparum* has the ability to utilize several invasion pathways involving different ligands and receptors. The parasite ligand – host receptor interactions during erythrocyte invasion for different *Plasmodium* species is discussed in the following sections.

Step 1: Initial attachment of the merozoite to the erythrocyte surface

The first step of erythrocyte invasion is known as initial attachment, during which the merozoite attaches to the erythrocyte through any part of its surface and most likely involves the merozoite surface coat filaments. Video microscopy has shown that these merozoite–erythrocyte contacts coincide with heaving perturbations of the red cell membrane, which makes these interactions appear dynamic (Dvorak, 1975; Gilson and Crabb, 2009; Esposito, 2010; Tiffert, 2014; Crick, 2014). Initial attachment is reversible, and the parasite is not committed for invasion. Thus, the merozoite can detach and adhere to another erythrocyte. It is reasonable to believe that merozoite surface proteins (MSPs) mediate this initial attachment. A number of merozoite surface proteins have been identified that are constitutively expressed on the outer surface of the merozoite and are implicated to play a role in invasion (Table 3.1); these are described below. However, it is not yet clear which proteins are involved in initial attachment because a number of MSPs as well as several adhesive proteins are secreted to the merozoite surface and bind with the erythrocyte, including MSP-1 and MSP-DBLs, the Duffy binding–like (DBL) and reticulocyte binding–like (RBL) family of proteins, which are discussed later.

Merozoite surface proteins

The thick fibrillar coat on the outer surface of the merozoite is composed of a number of integral and peripheral membrane proteins known as the merozoite surface proteins (MSPs). Eight MSPs are membrane bound through a glycosylphosphatidylinositol (GPI) anchor, and several more peripheral MSPs (Figure 3.4) are either tightly associated with the GPI-anchored MSPs or are largely soluble proteins of the parasitophorous vacuole (Gaur, 2004; Kadekoppala and Holder, 2010).

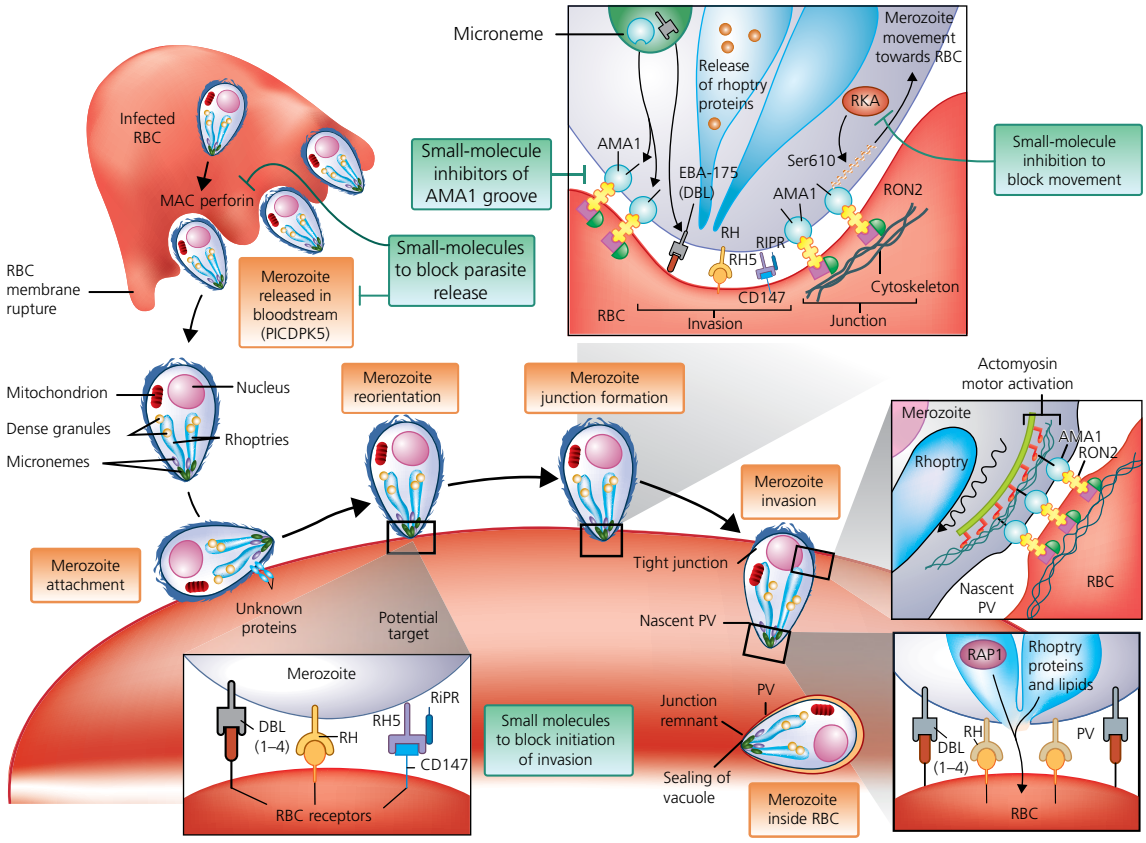


Figure 3.3 Steps of erythrocyte invasion by *P. falciparum* merozoites and potential strategies to block invasion. The different steps constituting the merozoite invasion process in erythrocytes are depicted. Merozoites undergo egress from an infected erythrocyte and are released into the blood stream. The calcium-dependent protein kinase 5 (CDPK5) plays an important role in egress. Thereafter, the merozoite attaches to the erythrocyte membrane, apically reorients, and forms a junction. A number of DBL and RH proteins mediate attachment by binding with their respective erythrocyte receptors. AMA-1 binds with RON2, which traverses the host red cell surface and associates with RON4/5 that are localized in the erythrocyte cytosol.
Source: Miller LH, Ackerman HC, Su XZ, Wellems TE. 2013. Malaria biology and disease pathogenesis: insights for new treatments. *Nature Medicine.* 19(2):156–167.

The GPI-anchored MSPs include MSP-1, MSP-2, MSP-4, MSP-5, MSP-10, and Pf92 (Gaur, 2004; Cowman, 2012), of which MSP-1 is probably the most extensively studied. The peripheral MSPs include MSP-3, MSP-6, MSP-7, MSP-8, and MSP-9 (Gaur, 2004). The interactions between the MSPs and other parasite proteins are described below.

MSP-1/6/7 complex

The most abundant merozoite surface proteins are the multiprotein complex of three noncovalently linked MSPs (MSP-1, MSP-6, MSP-7). MSP-1 is a 190-kDa protein that undergoes proteolytic processing to yield four polypeptide fragments during merozoite maturation at the schizont stage of the blood-stage life cycle (Figure 3.5). The four fragments that are present in the MSP-1/6/7 complex include an 83-kDa N-terminal fragment ($MSP\text{-}1_{83}$), two internal fragments of 30 kDa ($MSP\text{-}1_{30}$) and 38 kDa ($MSP\text{-}1_{38}$), and a C-terminal 42-kDa fragment ($MSP\text{-}1_{42}$) (Holder and Freeman, 1984; Holder, 1987).

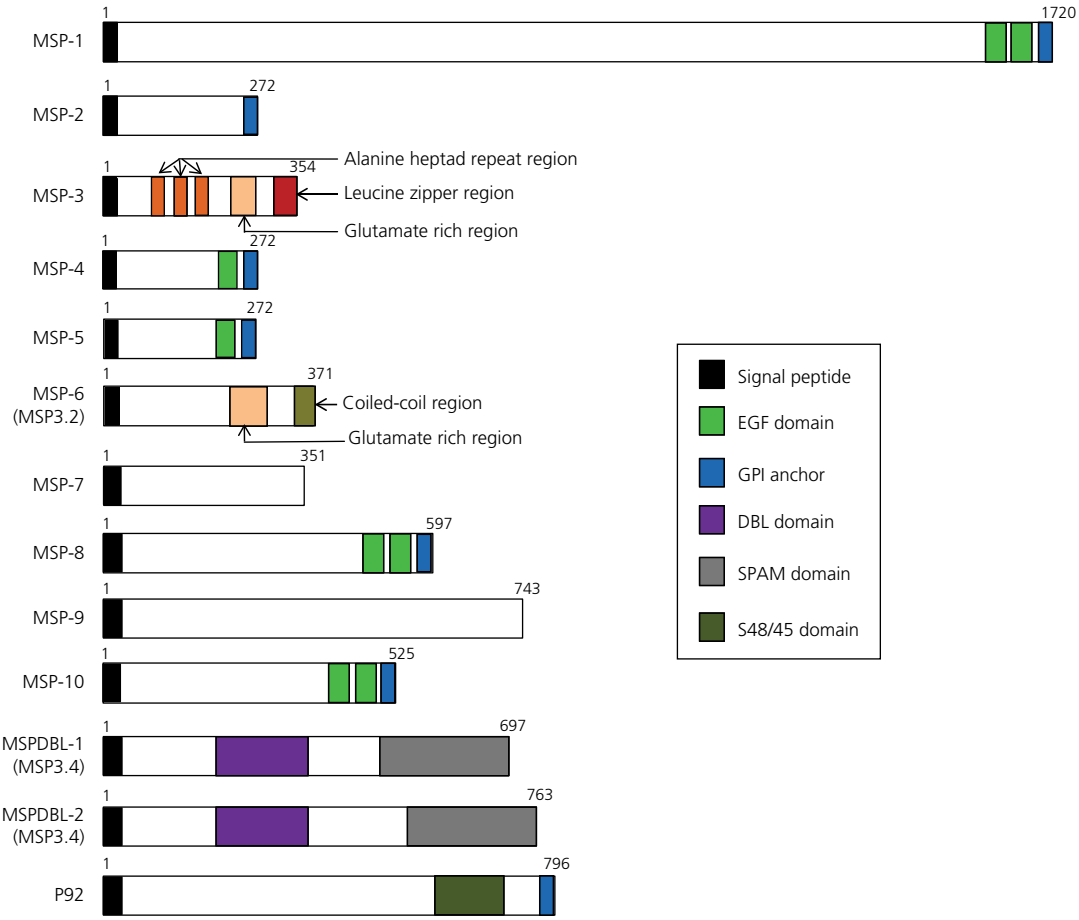


Figure 3.4 Structural features of the MSP family of proteins are depicted in the schematic diagram. All MSPs possess a signal peptide. MSP-1/-2/-4/-5/-8/-10 are GPI-anchored, and all of them except MSP-2 comprise EGF domains. MSP-3 possesses alanine heptad repeats, a glutamate-rich region, and a leucine-zipper region. The closely related MSP-6 possesses a glutamate-rich region and a coiled-coil region at its C-terminal end. Pf92 is a GPI-linked member of the 6-cysteine protein family that is localized on the merozoite surface. Two DBL domain–containing MSPs have been identified: MSPDBL-1 and MSPDBL-2.

MSP-1 has attracted a lot of attention since it was first discovered in *P. knowlesi* (David, 1984) and other *Plasmodium* species (Holder and Freeman, 1982; Holder, 1985a; Holder, 1988). Despite significant efforts to understand its structure, biochemistry, and immunoepidemiology, the precise functional role of MSP-1 is still not understood. Earlier it was well reported that MSP-1 is expressed as a large precursor protein during the late schizont stages of the blood-stage life cycle and undergoes proteolytic processing during egress and invasion to produce the four fragments. The last decade has, however, shed more light on the details of this process, and now we know that MSP-1 undergoes an initial primary processing step during egress in which the subtilisin-like protease SUB1 cleaves the protein into the four fragments followed by a secondary processing step in which the 42-kDa C-terminal fragment ($MSP\text{-}1_{42}$) is cleaved by another subtilisin-like protease, SUB2, to yield the two smaller fragments, $MSP\text{-}1_{33}$ and $MSP\text{-}1_{19}$ (Yeoh, 2007; Child, 2010). The second processing step

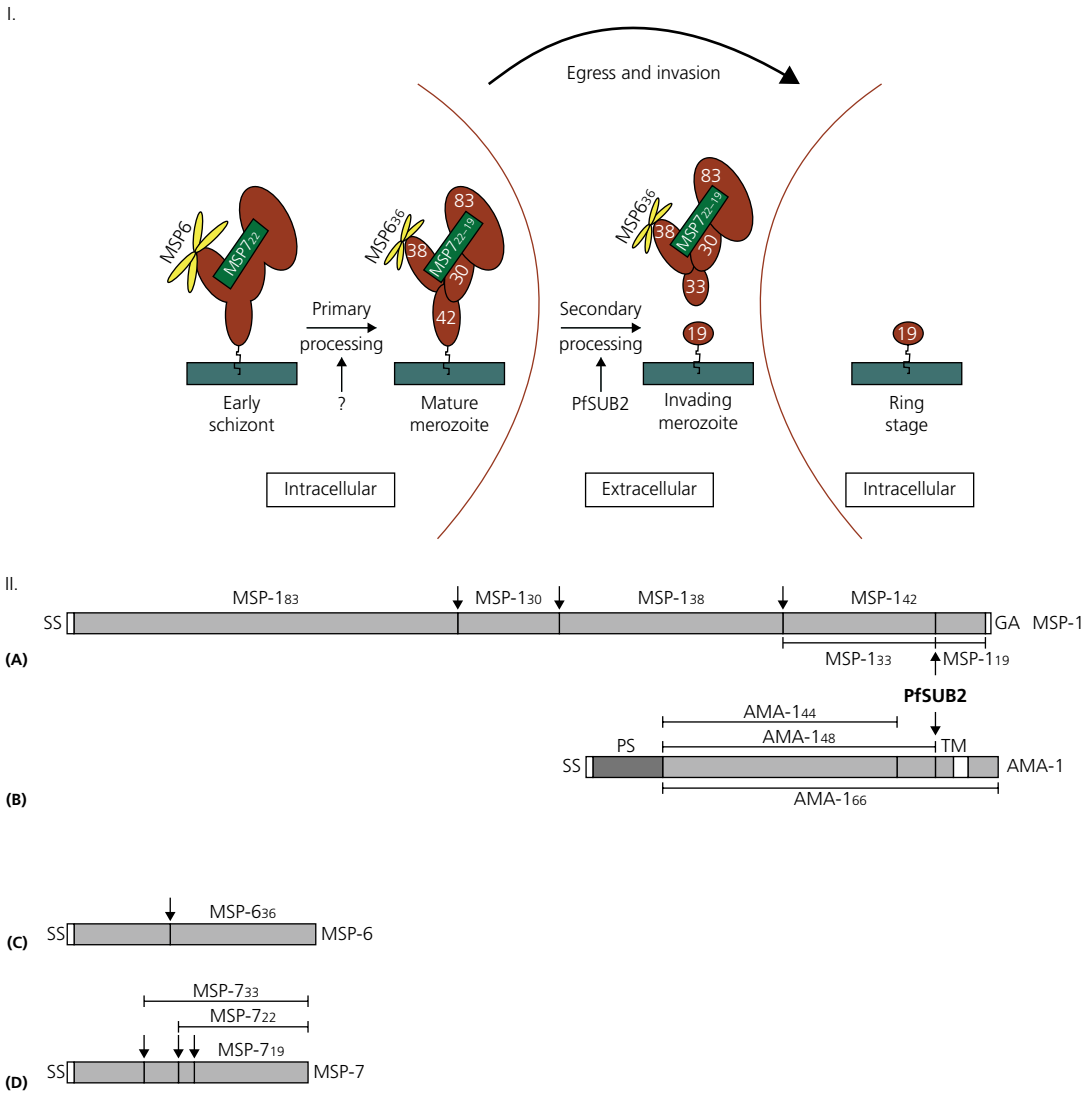


Figure 3.5 Processing of the MSP-1/6/7 Complex. Part I. Proteolytic processing of the MSP1/6/7 complex. Biosynthesis of all three MSPs begins at the start of schizogony (nuclear division). As indicated, the complex is a stoichiometric assembly of the various components, except that the MSP-6 precursor binds as a tetramer (Kauth 2006). Primary processing converts it to MSP-6_{36}. The MSP-7 precursor undergoes an early processing step during secretory transport (Pachebat 2007) to produce MSP-7_{33} (green), the form that binds to the MSP-1 precursor (brown). Primary processing converts MSP-7_{33} to both MSP-7_{22} and MSP-7_{19}. The various MSP-1 processing products are numbered for clarity. Secondary processing, which occurs following egress and probably at the point of invasion, involves further cleavage within MSP-1_{42} to produce MSP-1_{33} and MSP-1_{19}. The latter is carried into the newly invaded erythrocyte on the merozoite surface (to form a so-called ring-stage parasite), whereas MSP-1_{33} is shed along with the remaining MSP-1/-6/-7 complex. Secondary processing is mediated by a membrane-bound protease called PfSUB2, whereas the protease(s) responsible for primary processing has been hitherto unknown (indicated by a question mark).

Part II. Primary structure and processing of *P. falciparum* 3D7 MSP-1, MSP-6, MSP-7 and AMA-1. SS, signal sequence; GA, GPI anchor; PS, pro-sequence; TM, transmembrane domain. (A) Outline of the MSP-1 precursor.

occurs right at the time of merozoite invasion and results in the release of the soluble MSP-1_{33}, whereas the C-terminal GPI-anchored MSP-1_{19} remains tethered to the merozoite surface as it enters the host erythrocyte. MSP-1_{19} possesses two epidermal growth factor (EGF)-like domains (Blackman, 1991), and following invasion it homes to the food vacuole and is thus the first marker for the biogenesis of the food vacuole (Dluzewski 2008). Interestingly, newly synthesized MSP-1 remains excluded from the food vacuole, which has led to the inference that MSP-1_{19} follows the classical lysosome-like clearance pathway. Thus, it appears that MSP-1_{19} may play a significant role in the biogenesis and function of the food vacuole throughout the intraerythrocytic phase.

MSP-1 is an essential parasite protein, which like other GPI-anchored MSPs has been refractory to gene disruption (knockout) by genetic manipulation (Sanders, 2006). A number of reports have suggested that MSP-1 binds human erythrocytes. Initially it was reported that full-length MSP-1 binds erythrocytes in a sialic acid–independent manner (Perkins and Rocco, 1988), followed by a report that a 115–amino acid region within MSP-1_{38} binds erythrocytes and elicits potent invasion-inhibitory antibodies (Nikodem and Davidson, 2000). More recently, it has been shown that MSP-1_{19} binds with Band 3 in a co-complex with MSP-9 (Li, 2004).

MSP-1 is known to be present on the merozoite surface in the form of a complex with other parasite molecules (Figure 3.5). It was first reported to form a complex with MSP-6 and MSP-7, which are 36-kDa and 22-kDa proteins, respectively, that were identified from the shed MSP-1 complex from the culture supernatants of cultured *P. falciparum* parasites (Pachebat, 2001; Kauth, 2006). MSP-6 was later on shown to be a member of the MSP-3 family of proteins that are encoded by a group of genes located together on chromosome 10 (Singh, 2009). MSP-6 and the MSP-3 family are described later.

MSP-7 is a 351-amino acid protein containing an N-terminal signal sequence followed by an acidic region and a conserved C-terminal structured region (Pachebat, 2001). MSP-7 is produced as a 48-kDa protein during late schizogony. It appears that the association of MSP-7 with MSP-1 occurs soon within the pre-Golgi compartment of the secretory pathway (Pachebat, 2007) and is crucial for its translocation and anchorage to the merozoite surface. There is no detection of MSP-7 as a soluble protein in the parasitophorous vacuole, suggesting that its interaction with MSP-1 is crucial for their translocation to the merozoite surface (Trucco, 2001).

It has also been reported that MSP1/6/7 is part of a larger protein complex on the merozoite surface during invasion that also comprises ten more proteins: MSP-3, MSP-9, RhopH1, RhopH3,

Figure 3.5 (Continued) The grey arrows indicate the sites of primary processing of the precursor protein into its major subunits MSP-1_{83}, MSP-1_{30}, MSP-1_{38}, and MSP-1_{42} (Stafford 1994, Koussis 2009). A secondary proteolytic cleavage mediated by PfSUB2 (black arrow) occurs during invasion, cleaving MSP-1_{42} into MSP-1_{33} and MSP-1_{19}. (B) AMA-1 is synthesized as an 83 kDa precursor protein containing a C-terminal transmembrane domain (TM). After targeting to the micronemes the N-terminal pro-sequence (PS) is removed, resulting in AMA-1_{66}, which appears at the merozoite surface at the time of schizont rupture. During invasion AMA-1_{66} is proteolytically cleaved by PfSUB2 (black arrow) resulting in release of AMA-$1_{48/44}$ (Howell 2003). MSP-6 (C) and MSP-7 (D) are peripheral merozoite surface proteins, membrane-bound through non-covalent associations with MSP-1. MSP-6 is processed into MSP-6_{36}. MSP-7 is initially cleaved into MSP-7_{33} (Pachebat 2007). Around the time of merozoite release from the newly ruptured schizont, MSP-7_{33} is further cleaved into MSP-7_{22} and MSP-7_{19} (Pachebat 2001, 2007). Source: (A) A multifunctional serine protease primes the malaria parasite for red blood cell invasion. Koussis K, Withers-Martinez C, Yeoh S, Child M, Hackett F, Knuepfer E, Juliano L, Woehlbier U, Bujard H, Blackman MJ. The EMBO Journal (2009) 28,725-735. (B) Antibodies against multiple merozoite surface antigens of the human malaria parasite *Plasmodium falciparum* inhibit parasite maturation and red blood cell invasion. Woehlbier U, Epp C, Hackett F, Blackman MJ, Bujard H. Malaria Journal (2010) 9:77.

RAP-1, RAP-2, and two RAP domain–containing proteins (Ranjan, 2011). MSP-1 and MSP-9 have also been reported to interact with each other (Li, 2004). Although these are all interesting observations, a better understanding of their physiological significance with respect to merozoite biology or erythrocyte invasion requires further investigations.

It has been reported that two peripheral merozoite surface proteins containing DBL domains, MSPDBL-1 and MSPDBL-2, are part of the MSP-1/6/7 large complex and directly associate only with MSP-1 (Lin, 2014). The MSPDBL-1 (PF3D7_1035700) and MSPDBL-2 (PF3D7_1036300) proteins are members of the MSP-3 family (Singh, 2009; Hodder, 2012), which comprise the characteristic cysteine-rich DBL domain and have been reported to be localized to the surface of the merozoite (Wickramarachi, 2009). The presence of the DBL adhesive domains does impart erythrocyte-binding properties to both MSPDBL-1 and MSPDBL-2 that have been reported to bind erythrocytes, although their specific receptors still remain to be defined. It is highly likely that the MSPDBLs play a role in the initial attachment of the merozoite with the target erythrocyte, which has been further substantiated by the observation that antibodies against MSPDBL-1 inhibit erythrocyte invasion (Sakamoto, 2012). However, they do not possess transmembrane domains or glycosylphosphatidylinositol anchors and hence are believed to be secured on the merozoite surface through their association with other parasite proteins. It has been elegantly demonstrated that the MSPDBL proteins interact with the MSP-1/6/7 complex. In this multiprotein complex, the MSPDBLs directly interact with the MSP-1 complex and do not interact with MSP-6 (Lin 2014). Thus, the MSP1/6/7 complex acts as a platform to secure the MSPDBLs on the merozoite surface and facilitate their binding to the erythrocyte during invasion. Further structure–function insights into the formation of the MSPDBL–MSP1 complex would be highly beneficial in understanding their role during invasion and would support efforts to develop vaccines that target this major multiprotein complex.

MSP-3 family

MSP-3 is one of the soluble merozoite surface proteins that was first discovered as a target of a protective immune response known as antibody-dependent cellular inhibition (ADCI) (McColl, 1994; Oeuvray, 1994). Antibody responses to merozoite surface proteins are known to be associated with protective immunity against malaria. Some antibodies mediate their protective role through complement-mediated lysis, and others act through cooperation with Fc receptor–bearing cells. For example, cytophilic antibodies (IgG1 and IgG3) against MSP-3 facilitate killing by monocytes (Bouharoun-Tayoun, 1995). This mechanism of monocyte-dependent growth inhibition, also known as ADCI, is mediated by soluble components released by the monocytes that inhibit intraerythrocytic development of the parasite.

Antibodies to antigens other than MSP-3, such as MSP-1 block 2 (Galamo, 2009), MSP-2 (Flueck, 2009), glutamate-rich protein (GLURP) (Jogdand, 2012), and serine repeat antigen 5 (SERA5) (Yagi, 2014) also exhibit ADCI effector activity. These antigens are also not directly secured on the merozoite surface and are tethered to the surface through associations with other proteins in the form of complexes. Thus, MSP-3 is considered a malaria vaccine candidate, which is supported by both field studies and clinical trials that are discussed in later chapters.

Structurally, MSP3 is highly conserved, with 12 copies of a degenerate heptad repeat (AXXAXXX) in the N-terminal region and with a glutamic acid-rich domain and a leucine zipper motif in the C-terminal region (McColl, 1994). The presence of the heptad repeats is consistent with the formation of an intramolecular coiled coil (Mulhern, 1995; Imam, 2014). Although the C-terminal region has been implicated in MSP-3 oligomerization (Burgess, 2005), its role in eliciting protective antibody responses is not yet clear. MSP-3 has been implicated to play a role in the trafficking of MSP-9 (ABRA) to the merozoite surface (Mills, 2002), which is discussed later in the section on

MSP-9. There thus appears to be a significant cross-talk between the merozoite surface proteins during invasion, which is exemplified by the MSP-1/6/7/MSPDBL complex (Lin, 2014) as well as the MSP-1/MSP-9 co-ligand complex.

MSP-3 was subsequently found to be part of a larger multigene family that is located on chromosome 10 (Singh, 2009). Bioinformatics analysis first identified MSP-6 as having significant sequence similarity with MSP-3 within its C-terminal region and also sharing a signature sequence near the N-terminus (NLRNA/G) (Singh, 2005). This signature sequence was found to be the hallmark of eight MSP-3 like paralogs that were all identified to be located contiguously on chromosome 10. In the new nomenclature of the family, MSP-3 is known as MSP-3.1; MSP-6 is known as MSP-3.2; H101 is renamed MSP-3.3; H103 is renamed MSP-3.7; and the remaining are MSP-3.4, MSP-3.5, MSP-3.6, and MSP-3.8 (Singh, 2009). The fact that these genes are all contiguously located on the same chromosome strongly suggests that these genes may have evolved due to gene duplication events, a phenomenon that has been reported for several other genes. Whereas all eight paralogs share the signature sequence on the basis of which they were all identified, only six of them—excluding MSP-3.5 and MSP-3.6—share a similar sequence organisation in their C-terminal regions (Singh, 2009). Within the *P. falciparum* species, these six genes displayed a very high degree of sequence conservation in their C-terminal regions among 36 field strains from different parts of the world, which is rare for genes encoding surface proteins and thus strongly suggests an important physiological role of these regions in the biology of the parasite. Naturally acquired antibodies against the C-terminal regions of all the MSP-3 family of proteins exhibit cross-reactivity and are effective in parasite killing as measured in *in vitro* ADCI assays (Singh, 2009).

MSP-2

Merozoite surface protein 2 (MSP-2) is another GPI-anchored 30-kDa protein on the surface of *P. falciparum* merozoites (Gerold, 1996) that has been under development as a potential component of a blood-stage vaccine, Combination B (Genton, 2002). Unlike MSP-1 and AMA-1, MSP-2 lacks multiple intramolecular disulfide bonds with limited knowledge of its three-dimensional structural characteristics that are crucial for eliciting protective immune responses. MSP-2 is highly polymorphic, especially in its central variable region, with its N- and C-terminal domains being conserved (Smythe, 1991). Based on these sequence differences, MSP-2 alleles have been categorized into two families that are typified by the 3D7 and FC27 alleles (Smythe, 1991). Both dimorphic variants of MSP-2 comprise regions of low complexity and biased amino acid composition (unusually hydrophilic and deficient in hydrophobic residues). These unique characteristics are consistent with studies that have shown full-length MSP-2 being an intrinsically unstructured protein (IUP) that can form amyloid fibrils in its recombinant form (Adda, 2009).

The fact that recombinant MSP-2 forms amyloid fibrils in solution implies that native MSP-2–based amyloid fibrils might constitute the fibrillar merozoite surface coat. However, the conformation of native MSP-2 on the merozoite surface may be distinct from that of monomeric recombinant MSP-2 due to the constraints enforced by membrane-anchoring and other protein interactions involved in the formation of the merozoite surface coat (Adda, 2009). The tendency of MSP-2 to form fibrils in solution is significant for its development as a vaccine candidate, because the immunogenic characteristics of the soluble monomeric form versus the fibrils are expected to be different (Chauhan, 2010). Thus, our incomplete knowledge of the structure of native MSP-2 is a primary obstacle in the development of an MSP-2–based malaria vaccine. Hence, it is crucial that further research efforts be undertaken to solve the structural conformation of native MSP2 on the merozoite surface as well as to elucidate the mechanism of fibril formation.

MSP-4/5

MSP-4 is 40-kDa GPI-anchored essential parasite protein, which interestingly has a single EGF-like domain in the carboxyl terminus that exhibits a spacing pattern of cysteine residues as observed in MSP-1 but with different intervening residues (Marshall, 1997). The EGF-like domain is crucial for the native structural conformation of MSP-4 because its disruption severely affects antibody reactivity even in parts of MSP-4 that are not involved in forming disulfide bonds (Wang, 1999). The protein is highly immunogenic in small animals as well as humans. The reactivity of human antibodies against MSP-4 is also highly dependent on the correct folding of the EGF-like domain. The function of MSP-4 is not yet understood; however, the fact that it cannot be genetically knocked out as for other GPI-anchored MSPs does imply an essential function (Sanders, 2006).

The *P. falciparum* MSP-4 gene is located in a 10-kb region of chromosome 2 that also encompasses the coding region of MSP-2 as well as that of a parasite protein, which shares features of the MSP family, and has been termed MSP-5 (Marshall 1998). The MSP-4 and MSP-5 genes are closely related, because they share several properties. Their gene structure is similar, with two exons; they code for proteins of the same length that possess hydrophobic signal peptides, GPI anchorage signals, and a single carboxy EGF-like domain (Marshall 1998). The close proximity in location of the genes and similar protein characteristics strongly suggests that both genes have arisen from a gene duplication event. MSP-5 is transcribed in the asexual stages and further translates in to a 40-kDa protein that is localized on the merozoite surface (Marshall, 1998).

In sharp contrast to several blood-stage antigen genes, *msp4* and *msp5* are highly conserved, with no polymorphisms reported for MSP-5 among several *P. falciparum* isolates (Wu, 1999). Only a limited antigenic diversity has been reported for MSP-4, with nine residues exhibiting polymorphisms (Benet, 2004). It is not well understood whether this conserved nature is a reflection of an essential function or a lack of immune pressure.

Further studies have demonstrated the vaccine efficacy of *Escherichia coli*–expressed recombinant MSP4/5 in protecting BALB/c mice against a lethal *Plasmodium yoelii yoelii* YM blood-stage challenge (Goschnick, 2004). The protection was greatly increased through a combination of MSP4/5 and MSP119 (Kedzierski, 2002). All these features described above suggest MSP-4/5 as attractive candidates for inclusion in a multiantigen vaccine.

MSP-8

A bioinformatics analysis in which the *P. falciparum* genome was searched for putative genes encoding GPI-anchored proteins with double EGF-like domains at the C-terminus led to the identification of two more genes of the MSP family, *MSP-8* and *MSP-10* (Black, 2001; Black, 2003). PfMSP-8 is a 597–amino acid protein that shares features with those of PyMSP-8 (Black, 2001) and that, like other *P. falciparum* proteins, also undergoes proteolytic processing at both the trophozoite and schizonts stages to yield fragments of different molecular masses (98 kDa, 50 kDa, 25 kDa, and 19 kDa) (Black, 2001). Similar to MSP-1, it was proposed that MSP-8 was localized on the surface of free merozoites, and the presence of double EGF-like domains suggested that MSP-8 might also be a potent target of naturally acquired immunity as observed for MSP-1_{19}. In fact, a genetic manipulation study involving allelic replacement of the PfMSP-1 EGF domains with that of PbMSP-8 showed that the chimeric parasite line was able to invade erythrocytes and grow with the same efficiency as the wild-type parasite line (Drew, 2004). The demonstration of such a functional complementarity suggested that similar double EGF-like domains might support evasion of MSP-1_{19} invasion-inhibitory antibodies.

However, a later study has shown that surprisingly, *P. falciparum* MSP-8 is expressed during the ring stages and is not present on the surface of merozoites (Drew, 2005). In this regard, MSP-8 is a unique GPI-anchored protein to be expressed during the early stages of the intracellular erythrocytic cycle.

It gets synthesized as a mature 80-kDa parasite protein that is processed to a C-terminal 17-kDa polypeptide comprising the two EGF-like domains. Immunofluorescence and membrane-association studies have shown that MSP-8 is initially located during the ring stage in the plasma membrane of the parasite (Drew, 2005). During the late schizont stages, the processed 17-kDa fragment is present in the food vacuole. The MSP-8 gene could be successfully disrupted, implying that it does not play an essential role (Drew, 2005). This feature again makes MSP-8 an exceptional member of the GPI-anchored MSP family because most GPI-anchored proteins are refractory to genetic manipulation. Thus, although MSP-8 does not appear to a be a viable vaccine target primarily due to its intracellular nature, based on the time of its expression it might play a role in protein trafficking across the parasitophorous vacuole membrane (PVM) during the erythrocytic stages of the life cycle, which needs further investigation.

MSP-9 (ABRA)

MSP-9 or ABRA (acidic and basic residues antigen) is another highly conserved parasite protein that is present on the surface of merozoites as well as in the parasitophorous vacuole within the infected erythrocytes (Stahl, 1986; Kushwaha, 2000). It has a molecular mass of 101 kDa and comprises several repeats of acidic and basic amino acids. A significant aspect of ABRA is its presence in the merozoite clusters formed during parasite egress (Lyon, 1986). Its antibodies are thus associated with a blockade of merozoite dispersal, leading to a reduced parasitemia. The low parasite densities are considered an indicator of protective immunity. Furthermore, ABRA is highly conserved among various *P. falciparum* clones (Weber, 1988) and shares partial homology with an extracellular cysteine protease of *Trichomonas vaginalis*, another pathogenic protozoan parasite (Garber, 1993). It exhibits chymotrypsin-like protease activity (Nwagwu, 1992), which resides in its N-terminal region and suggests a putative role of MSP-9 in protease-mediated processes during schizont egress and merozoite invasion.

MSP-9 is localized on the merozoite surface, and MSP-3 has been implicated to play a role in its trafficking to the parasite surface (Mills, 2002). MSP-3 comprises three stretches of heptad repeats, a glutamic acid–rich region and a putative leucine zipper sequence at the C-terminus (McColl, 1994). It has been demonstrated that disruption of the *msp3* gene at the C-terminus produces a truncated form of MSP-3 that is specifically deficient in the putative leucine zipper sequence and was observed not to localize to the parasitophorous vacuole or interact with the merozoite surface as reported for wild-type MSP-3 (Mills, 2002). Furthermore, these parasites expressing the truncated form of MSP-3 did not express the acidic–basic repeat antigen (ABRA) on the merozoite surface, and the merozoites invaded erythrocytes with a lower efficiency (Mills, 2002). Thus, it appears that MSP-3 is not entirely essential for sustaining blood-stage parasites. However, its leucine zipper region plays a crucial role in its trafficking as well as that of ABRA, which are in turn critical for complete fitness of the merozoite to invade erythrocytes with full efficiency.

It was further demonstrated that ABRA binds erythrocytes and specifically interacts with band 3 as its receptor (Kushwaha, 2002; Li, 2004). The receptor-binding domain of ABRA was mapped to its highly conserved N-terminal cysteine-rich region (Kushwaha, 2002). Co-immunoprecipitation studies showed that MSP-9 forms a co-ligand complex with MSP-1 that bound band 3 (Li 2004). Although the precise mechanism through which the MSP co-ligand complex interacts with band 3 is yet not completely understood, it has been proposed that MSP-1_{42} and MSP-9 could bind to two different regions or faces of a single 5ABC segment of band 3 (Model I), or that initially only one may bind with a single 5ABC domain and later an equilibrium state is attained in which both MSPs might interact at the same time with an independent 5ABC domain (Model II) (Li, 2004). It has been reported that the latter model appears more favorable in light of the fact that native band 3 exists either in the form of dimers or tetramers on the erythrocyte surface (Li, 2004). It is thus

possible that the merozoite could attach with the erythrocyte through either MSP1-band 3 or MSP9-band 3 interactions, which may be redundant interactions in which different parasite molecules engage with the same receptor molecule, further leading to involvement of key ligand–receptor interactions that complete the process of attachment and apical reorientation of the merozoite on the erythrocyte surface.

Full-length recombinant ABRA (MSP-9) exhibiting functional protease activity has been successfully produced in *E. coli,* and its immunodominant B and T cell epitopes have been defined using synthetic peptides (Sharma, 1998). It was shown that some ABRA peptides were readily recognized by human IgG antibodies and at the same time activated the peripheral blood mononuclear cells from a large proportion of malaria-infected people in endemic regions. Some of these antibodies were observed to display potent inhibition of erythrocyte invasion by *P. falciparum* (Kushwaha, 2001). Therefore, based on its location and functional properties, ABRA (MSP-9) appears to be an attractive blood-stage malaria vaccine candidate that should attract more attention for clinical development.

MSP-10

MSP-10 was also identified through a bioinformatics analysis and was characteristically observed to contain two C-terminal epidermal growth factor (EGF)-like domains as observed for MSP-1 and MSP-8 (Black, 2003). The MSP-10 gene consists of a single 1572-bp open reading frame that encodes a 524–amino acid protein with a signal peptide and a GPI-anchorage site (Black, 2003). Interestingly, MSP-10 is unique, because its antibodies directed against the EGF-like domains exhibit a high specificity with no cross-reactivity with the EGF-like domains of MSP-1, MSP-4, MSP-5 or MSP-8. Like other MSPs, MSP-10 also undergoes processing as antibodies against different regions of the protein detect different fragments. Triton X-114 partitioning confirms that MSP-10 is associated with the detergent-enriched membrane fraction, and it has been reported to be expressed on the surface of the trophozoite and schizont stages as well as in free merozoites. MSP-10 has been shown to be recognized by human immune sera and exhibits limited polymorphisms in its two EGF-like domains. *P. falciparum* MSP-8 and MSP-10 gene polymorphisms are primarily nonsynonymous substitutions, a pattern that has been found to be consistent with a gene under positive selection (Pacheco, 2012). Some evidence for purifying selection was also reported for both MSP-8/MSP-10 orthologs in *P. cynomolgi*. Phylogenetic analysis has reported purifying selection for both MSP-8 and MSP-10 in the lineage leading to *P. vivax*. Antigens that exhibit purifying selection and appear to have evolved under strong functional constraints are poised to be potent vaccine candidates. A *P. yoelii* homologue of MSP-10 has also been identified, which allows us to test the vaccine potential of MSP-10 in an *in vivo* rodent malaria model (Pacheco, 2012).

In addition to merozoite surface proteins, there is a large repertoire of parasite adhesins in apical organelles (micronemes, rhoptries) that may also mediate initial attachment. These parasite ligand-host receptor interactions are discussed in great detail later in the chapter.

Step 2: Apical reorientation

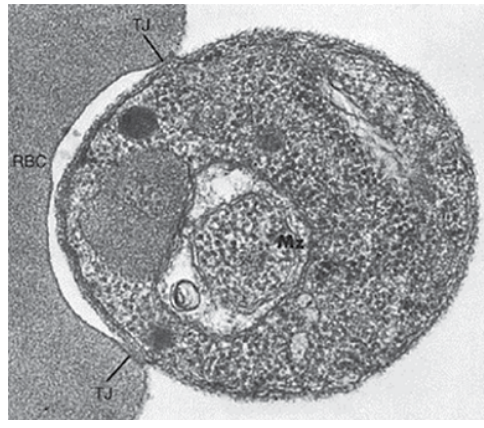
Initial attachment is followed by the second step of erythrocyte invasion, apical reorientation. Initial attachment occurs primarily through any part of the merozoite surface and mostly the apex of the merozoite is not directly apposed with the erythrocyte surface. Thus, it is imperative for the merozoite to undergo apical reorientation such that the apical organelles lined up at the apex are directly juxtaposed to the erythrocyte membrane. The specific molecular interactions involved in apical reorientation are poorly understood. It was initially suggested that apical membrane antigen 1 (AMA-1) might have a role in apical reorientation (Mitchell 2004). This inference was based on the observation that *P. knowlesi* merozoites attached normally to the erythrocyte surface but did not

undergo apical reorientation in the presence of a monoclonal antibody R31c2 against AMA-1 (Mitchell 2004). In contrast, Louis Miller's group showed that in the presence of the AMA-1 inhibitory monoclonal antibody 4G2, the cytochalasin-treated merozoites were able to apically reorient such that the apical end juxtaposed with the erythrocyte membrane (Srinivasan 2011). Thus, further in-depth studies would be required to decipher the molecular interactions that specifically mediate apical reorientation during invasion.

Step 3: Formation of the junction

A key characteristic step of erythrocyte or host cell invasion by apicomplexan parasites is the formation of the tight junction, an electron dense thickening at the parasite–host cell interface that completely commits the parasite to that particular host cell (Figure 3.6). The junction is a central structure formed during the invasion of apicomplexan zoites, which acts as a tight connection between the zoite and the host cell. As the parasite enters the host cell, the junction moves as a circumferential ring from the apical pole to the posterior end of the parasite and has thus been called the moving junction (MJ). The MJ is crucial for successful invasion because it is coupled with the actin–myosin motor and serves as a support to propel the parasite into the parasitophorous vacuole. In addition, the MJ is also believed to be involved in the formation of the PVM and in defining its biochemical composition. The MJ proteins act as a molecular sieve that selectively allows specific host plasma membrane proteins to be incorporated in to the PVM, such as glycophosphatidyl inositol (GPI)-anchored proteins or raft-associated transmembrane proteins, whereas type I transmembrane proteins are excluded.

Although the morphological description of the moving junction as an electron-dense thickening was first reported in the 1980s, we are just starting to understand its molecular composition. Initial experiments on *Toxoplasma gondii* identified four rhoptry neck proteins (RONs) involved in forming a complex with AMA-1 in the junction. The RONs, which are secreted at the apical tip, form a



TRENDS in Parasitology

Figure 3.6 Electron micrograph of merozoite invasion. Transmission electron microscopy image of a merozoite *(Mz)* contacting an erythrocyte *(E)* during the process of invasion. The apical organelles in the merozoite (rhoptry, microneme, and dense granules) are shown. The erythrocyte membrane is thickened (15 nm) at the attachment site marked by the arrow, which represents the junction (magnification × 54,000). The formation of the junction commits the merozoite to invade the specific erythrocyte. *Source:* Proellocks NI, Coppel RL, Waller KL. 2010. Dissecting the apicomplexan rhoptry neck proteins. *Trends Parasitol*. 26:297–304.

characteristic ring structure that colocalizes with the MJ during invasion. These results were later confirmed in *Plasmodium* and were found to be conserved across different apicomplexans, suggesting a vital and universal functional role in host cell invasion by the MJ in these parasites. The different components of the MJ are described below.

Apical membrane antigen 1

AMA-1 is one the most well characterized parasite molecules. It was first identified in *P. knowlesi* and found to be highly conserved across different species of *Plasmodium* as it has been reported to play a central role in the biology of apicomplexans. It is a Type I integral membrane protein with a short cytoplasmic tail that translocates from the micronemes to the merozoite surface at time of host cell invasion. AMA-1 is an essential protein for *P. falciparum,* because no knockout of the gene has been reported; however, knockouts have been reported in *P. berghei*, which do suggest that its function might not be as essential.

P. falciparum AMA-1 (PfAMA-1) is produced as an 83-kDa precursor protein that gets cleaved releasing an N-terminal prodomain (Narum and Thomas 1994; Howell 2001), whereas in other *Plasmodium* species, AMA-1 is produced as a 66-kDa protein. Like several parasite proteins, AMA-1 also undergoes proteolytic processing to yield the 44-kDa and 48-kDa fragments following translocation to the merozoite surface (Howell 2001; Dutta 2003; Howell 2003).

The ectodomain of AMA-1 contains 16 cysteine residues, which are conserved throughout all *Plasmodium* species (Hodder 1996). The disulfide bonds between these cysteine residues produce three subdomains that constitute the extracellular region of AMA-1 (Hodder 1996). The crystal structures of *P. vivax* AMA-1 and *P. falciparum* AMA-1 domains have been solved (Figure 3.7) and have identified a hydrophobic pocket formed by two PAN domains that interacts with RON proteins (Bai 2005; Pizarro 2005; Coley 2007). AMA-1 derived from *Plasmodium* species and *T. gondii* are structurally conserved.

Antibodies against PfAMA-1 have potent invasion-inhibitory activity, and it has been considered a leading blood-stage vaccine candidate. Unfortunately, this inhibition is strain specific, and AMA-1 exhibits a high degree of antigenic polymorphism that mediates immune escape. As a result, AMA-1 has failed to elicit protection in field efficacy trials. Further modifications and novel strategies are being developed to harness the vaccine potential of AMA-1, which is described in greater detail in the specific chapter on malaria vaccine development. Thus, although AMA-1 has gathered a lot of interest in the field, its precise role in erythrocyte invasion has been unknown for a long time and has been elucidated only recently. A number of studies both in *Toxoplasma* and *Plasmodium* species have shown that AMA-1 interacts with the RON proteins, which localizes to the moving junction, which is discussed in greater detail in the next section.

Rhoptry neck proteins and their interaction with AMA1

The first insights into the interaction of AMA1 with the RON complex came from studies in *T. gondii* that showed that RON2/4/5/8 constitute a complex that co-immunoprecipitates with AMA-1 and colocalizes to the MJ during invasion. This interaction was later observed in *P. falciparum* as well, and both PfAMA-1 and the PfRON proteins were localized to the MJ during invasion. All components of the MJ are conserved across apicomplexan parasites, with the exception of RON8.

RON proteins do not possess any specific conserved domains that could be ascribed a particular functional role. RONs 4, 5, and 8 are soluble proteins, whereas RON2 is a transmembrane protein that is predicted to have 2 to 4 hydrophobic helices and is the only RON protein that directly interacts with AMA-1. Reports have shown that the RON proteins are exposed on the cytoplasmic side of the host erythrocyte, which suggests that the RONs get inserted inside the host erythrocyte. The model suggested by Lebrun and coworkers is that RONs 4 and 5 are present toward the cytoplasmic

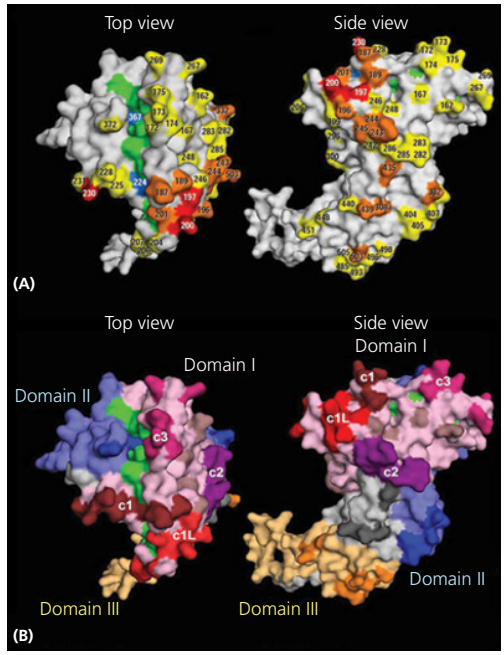


Figure 3.7 Structure of apical membrane antigen 1. Polymorphic amino acids shown on the apical membrane antigen 1 (AMA1) crystal structure. Polymorphisms are based on sequence data from *P. falciparum* infections acquired at a vaccine testing site in Mali, West Africa. *A,* Polymorphic residues are numbered and highlighted. *Yellow* and *blue* residues are dimorphic, *orange* residues are trimorphic, and *red* residues have four to six possible amino acids. Residues highlighted in *green* and *blue* make up the hydrophobic pocket hypothesized to be a binding site between AMA1 and the rest of the erythrocyte invasion machinery, with *blue* indicating polymorphic residues within the pocket. *B,* Conserved residues in AMA1 domains I, II, and III are highlighted, respectively, in *light pink, light blue,* and *light orange.* Polymorphic residues in domain I are highlighted in *dark brown* (c1), *red* (c1 and c1L), *purple* (c2), *dark pink* (c3), and *light brown* (not incorporated in a cluster). Polymorphic residues in domains II and III are highlighted, respectively, in *dark blue* and *dark orange. Light gray* residues are not part of any of the three major domains, and *dark gray* residues are polymorphisms within the interdomain region. *Source:* Takala SL, Coulibaly D, Thera MA, Batchelor AH, Cummings MP, *et al.* 2009. Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. *Science Translational Medicine.* 1:2ra5. (*See insert for color representation of this figure*.)

side of the host erythrocyte and remain associated with the N-terminal region of RON2, which traverses the host erythrocyte membrane, and its C-terminal domain, which is extracellular, interacts with AMA-1. Previously, the erythrocyte-binding activity of AMA-1 could not be established. It is now clear that during invasion, the parasite inserts RON2 into the host erythrocyte membrane, creating a receptor that binds with AMA-1. A conserved region of PfRON2 between two hydrophobic helices, referred to as RON2L, is implicated in binding with AMA-1.

Invasion inhibitory AMA-1 monoclonal antibody 4G2, which binds an epitope adjacent to the conserved hydrophobic pocket, blocks the interaction between AMA-1 and the RON complex. Similarly, AMA-1 binding peptides R1 or the RON2L peptide that binds the AMA-1 hydrophobic trough inhibit invasion by blocking the interaction of AMA-1 with the RON complex. Live video microscopy of merozoites in the presence of the R1 peptide or invasion inhibitory monoclonal antibody (mAb) 4G2 reveals that the invading merozoites successfully undergo attachment and apical reorientation but fail to invade target erythrocytes. Miller and coworkers have shown that in the

presence of the 4G2 antibody, merozoites were observed to bounce off the erythrocyte surface, which is consistent with the lack of junction formation. They further demonstrated that in the presence of the 4G2 antibody, cytochalasin D–treated merozoites apically reorient but fail to form a junction. It thus appears that formation of the AMA-1/RON complex occurs after apical reorientation, localizes junction formation, and precedes the release of rhoptry contents. These observations suggest that the interaction between AMA-1 and the RON complex defines a key step during erythrocyte invasion by malaria parasites. However, it has been demonstrated that it is possible to delete AMA-1 by genetic manipulation. Such AMA-1 knockout parasites can invade host erythrocytes, although they do so with lower efficiency due to poor rates of attachment. These observations suggest that parasites might have alternative mechanisms for invasion to enter host cells.

Linkage of the moving junction with the parasite motor

A large body of research is aimed at understanding the molecular mechanisms that underlie the motility of apicomplexan parasites, including the *Plasmodium* merozoites. One of the current models for apicomplexan motility suggests that an actin–myosin–based molecular motor drives movement of the parasite, including during invasion of host cells (Figure 3.8). On one side, the actin–myosin motor is linked to the cytoplasmic domains of adhesins, which bind to target host cells and constitute the moving junction. The rearward movement of the junction propelled by the myosin motor along the actin filaments drives the parasite forward. On the other side, the motor is anchored to the inner membrane complex, which is essential for the driving force generated by the myosin to be translated into parasite motility. A number of parasite proteins (MTIP, GAP-45, GAP-50) that secure the myosin A heavy chain in the cholesterol-rich, detergent-resistant membranes of the inner membrane complex have been identified (Figure 3.8).

Initiation of motility is probably triggered by a key intracellular signal (possibly Ca^{2+}) that initiates actin polymerization through the actions of Formin1 and Profilin. The actin filaments serve as a treadmill on which the myosin A filaments move and in the process generate an ATP-dependent directed force. The actin filaments were shown to be linked with the extracellular adhesin through a tetramer of the glycolytic enzyme aldolase in sporozoites, merozoites, and *T. gondii* tachyzoites. Thus, the force produced by the actin–myosin motor propels the parasite forward. Data have suggested that this model of motility based on links between parasite surface adhesins and the actin–myosin motor might not be the sole mechanism underlying parasite movement. Deletion of the gene encoding aldolase in *T. gondii* tachyzoites by genetic manipulation did not impair motility or host cell invasion (Shen and Sibley 2014). Similarly, deletion of genes encoding other components of the *T. gondii* actin–myosin motor, such as myosin, micronemal adhesin, MIC2, actin itself and GAP45, did not completely abrogate motility of host cell invasion, suggesting that there may be alternative mechanisms for merozoite motility and invasion that do not depend on the current model (Andenmatten 2013; Egarter 2014). Movement of the parasite into the parasitophorous vacuole requires that interactions between the extracellular adhesins and their respective host cell receptors be broken. This disengagement of ligand receptors is mediated by transmembrane proteases known as rhomboids, which cleave the adhesins in their transmembrane helices and subtilisin-like proteases, also known as sheddase, that are responsible for the shedding of two important surface antigens, AMA-1 and MSP-1.

TRAP family

The actin–myosin motor is linked with the host cell receptors through transmembrane adhesive proteins that primarily include a family of proteins defined by the thrombospondin-related anonymous protein (TRAP). Although the motor appears to be conserved among different apicomplexan parasites, the TRAP proteins are both species and stage specific, thus allowing the parasite to invade the respective host cells through a common substrate-dependent locomotion known as gliding motility. *P. falciparum* TRAP is a very well described molecule that plays a crucial role in sporozoite

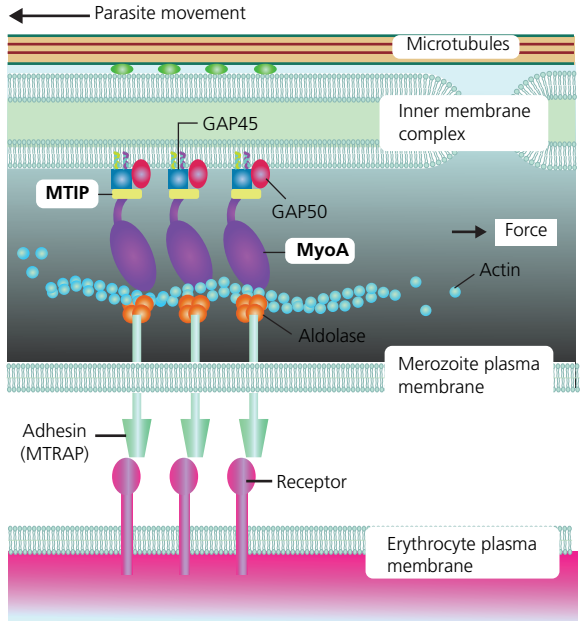


Figure 3.8 Invasion motor complex of *P. falciparum*. A schematic diagram of the invasion motor complex depicting a motility model of the malaria parasite during erythrocyte invasion. The sub-pellicular microtubules (top) are connected to the inner membrane complex (IMC). Myosin (MyoA) is anchored in the IMC through the MTIP-GAP45-GAP50 complex and walks on an actin treadmill that is formed by short actin filaments, which are polymerized at one end and depolymerized at the other. Actin is linked to the surface adhesins such as TRAP through a glycolytic enzyme, aldolase. The surface adhesins binds with the erythrocyte surface receptors (e.g., glycophorins, semaphorin-7A) to form an anchor with the substrate host erythrocyte. Myosin moves across the actin treadmill toward the end of actin polymerization. In the process, force is generated that is transmitted via the adhesins to the substrate to propel the parasite forward. The surface adhesins are eventually cleaved by the rhomboid proteases, which results in disengagement of the ligand–receptor interactions between the parasite and host erythrocyte substrate. *Source:* Thomas JC, Green JL, Howson RI, Simpson P, Moss DK, *et al.* 2010. Interaction and dynamics of the *Plasmodium falciparum* MTIP–MyoA complex, a key component of the invasion motor in the malaria parasite. *Molecular bioSystems.* 6:494–498.

motility and hepatocyte invasion. A number of TRAP homologues have been identified at different invasive stages including merozoites, which are described below, as well across different apicomplexan species. These characteristic proteins are known to contain one or more thrombospondin type-I repeat (TSR) domains, a von Willebrand factor (vWF)-like A domain in their extracellular region, and a tryptophan residue at the subterminal position of the cytoplasmic tail domain (CTD). The conserved nature of this family of proteins among apicomplexan parasites and their functional role in motility and invasion make them attractive molecules to study as well as target through different intervention strategies.

Merozoite-specific transmembrane adhesive proteins

The merozoite-specific TRAP homologue was identified and named MTRAP. It was shown to be localized in the micronemes, released and processed during invasion, and able to interact *in vitro* with aldolase—all key features similar to TRAP, suggesting it to be the blood-stage invasion adhesin that links the erythrocyte surface to the motor complex. Along with MTRAP, homologues of key motor

proteins such as the glideosome-associated proteins 45 and 50 (GAP45 and GAP50), are also present in *P. falciparum* merozoites and appear to function in erythrocyte invasion. MTRAP comprises two TSR domains and an acidic CTD with a sub-terminal tryptophan residue. It lacks a vWF-like A domain.

MTRAP is expressed primarily during the late schizont stages and gets translocated from the micronemes to the merozoite surface prior to invasion. During invasion, it undergoes two cleavage events, one in the extracellular region and another one in its transmembrane domain mediated by the rhomboid protease PfROM4. It has not been possible to genetically disrupt the MTRAP gene, suggesting its essential role during invasion and blood-stage parasite development. It was demonstrated that the acidic CTD of MTRAP bound with the actin-bound aldolase and that the mutation of the sub-terminal tryptophan residue led to an abrogation of this interaction. Although the erythrocyte-binding activity of native MTRAP has not yet been demonstrated, such activity was reported with the recombinant full-length protein produced in *E. coli.*

Following this study, another approach that had successfully identified basigin as the receptor for PfRH5 identified semaphorin-7A as the erythrocyte receptor of MTRAP. However, in the absence of any evidence that native MTRAP even binds erythrocytes, the significance of the MTRAP–semaphorin-7A interaction is not clear. This was further substantiated by the same study that showed that antibodies against MTRAP or semaphorin-7A did not exhibit any invasion inhibition.

Overall, several studies suggest that MTRAP is a merozoite-specific homologue of TRAP. MTRAP does not appear to be under positive selection and is highly conserved. This may primarily be due to the fact that MTRAP is not exposed to the host immune response by either being released very late during invasion or being incorporated cryptically in a complex such that it is not accessible to host antibodies. The observation that MTRAP antibodies fail to block erythrocyte invasion further supports this hypothesis.

P. falciparum thrombospondin-related apical merozoite protein

P. falciparum thrombospondin-related apical merozoite protein (PfTRAMP) is another TSR-containing merozoite protein that is implicated to play a role in erythrocyte invasion (Thompson 2004). It is localized at the apical end of merozoites and undergoes proteolytic cleavage, releasing it from the merozoite surface during invasion (Green 2006). PfTRAMP has been shown by confocal immunofluorescence microscopy and immunoelectron microscopy to be localized within the rhoptry bulb (Siddiqui 2013). It is expressed during all stages of intraerythrocytic development, with peak expression in late schizonts and merozoites (Siddiqui 2013; Le Roch 2003). In rings and trophozoites where rhoptries have not yet formed, PfTRAMP is found in the ER and Golgi as previously reported for rhoptry-associated membrane antigen (RAMA) and a 110-kDa rhoptry protein (Topolska 2004; Sam-Yellowe 1988). It translocates to the rhoptries in merozoites once they form in late schizonts. In addition, PfTRAMP was found to be secreted into the erythrocyte in whorl-like structures (Siddiqui 2013) as reported previously for other rhoptry proteins from *P. falciparum* (Riglar 2011) and similar to the evacuoles observed in case of *T. gondii* (Hakansson 2001).

Native and recombinant PfTRAMPs form disulfide-linked homodimers, both of which exhibit erythrocyte binding activity. The binding was resistant to treatment by neuraminidase, trypsin, and chymotrypsin, but it was sensitive to O-glycosidase, suggesting that PfTRAMP binds an O-linked carbohydrate moiety on the erythrocyte surface (Siddiqui 2013). As with MTRAP, the failure of efforts to knock out the gene that encodes TRAMP in *P. berghei* (Thompson 2004) suggests that it is essential for parasite survival. Antibodies against PfTRAMP individually exhibited modest invasion inhibition that could be enhanced in combination with EBA-175 antibodies.

The observations that MTRAP and PfTRAMP are essential, highly conserved parasite proteins strongly suggest that they play a significant physiological role in erythrocyte invasion *in vivo*, which needs to be further elucidated and defined.

New insights in to the molecular composition of the junction

The junction has been considered a traction point for the motor that links the parasite and host cell and comprises parasite proteins known to be conserved among apicomplexans. However, recent genetic data from both *Plasmodium* and *Toxoplasma* have challenged these preexisting concepts and provide a new point of view on the molecular basis of the junction.

Secondly, questions have also been raised as to the precise molecular composition of the junction and whether it comprises a conserved central core or primarily zoite-specific proteins. In *Plasmodium* merozoites, several proteins, including the DBL and RBL families of proteins, have been shown to be important for erythrocyte invasion and localized to the junction. Because these proteins are not conserved across the apicomplexan family and are expressed at specific stages, it is inferred that the junction comprising these proteins would be zoite specific. However, a conserved central core nature of the junction would suggest that the stage-specific proteins constitute adaptations to the invasion of specific target host cells. On the other hand, the motor binding TRAP family of proteins were considered primary components of the junction. However, there is no evidence to implicate the TRAP homologues in the formation of the junction during erythrocyte invasion.

Further in this regard, the role of AMA1 in formation of the molecular core of the junction has also been questioned based on the reports that genetic disruption of AMA-1 in *T. gondii* and *P. berghei* reduced the ability of the parasite to attach to the host cells but had no negative effect on formation of the junction or on their invasive ability. On the other hand, all efforts to genetically disrupt either RON2 or RON4 in both *T. gondii* and *P. berghei* have failed, strongly suggesting that the RON proteins play an essential role in junction formation and host cell invasion. In *P. falciparum*, a conditional knockdown of AMA-1 using the DiCre-loxP system had a negative effect on invasion rates, which was suggested to be due to a resealing defect of the vacuolar membrane. This implies that AMA-1 might have additional roles in the invasion process. One report showed that a *T. gondii* AMA-1 knockout parasite line exhibited an invasion efficiency that was 90% lower than that of the wild-type parasite line. However, the tachyzoites were observed to form the junction, and subsequent binding assays demonstrated that the RON protein had bound an AMA1-like second protein termed AMA-2. Further disruption of both AMA-1 and AMA-2 showed that *T. gondii* holds several AMA-1 homologues that could form multiple junctions and mediate invasion. It would be interesting to know whether such AMA1 homologues exist in *P. falciparum.*

Overall, it is interesting that many studies have challenged the current model of the junction and have led to a certain reassessment of the molecular mechanism underlying junction formation. Two main possibilities appear to be feasible at the moment. One is that the current model is incorrect and that AMA-1 has no role in junction formation and is only an erythrocyte-binding protein. This would suggest that the junction is formed through a distinct set of parasite molecules that have yet to be defined. The other possibility is that in wild-type parasites, AMA-1 and RON proteins are responsible for junction formation, but the parasite has redundant molecules that could compensate for the loss of AMA-1. Although the newest evidence is not conclusive enough to rule out the current model, it has produced interesting leads for further research efforts that would help in clarifying the molecular mechanisms underlying the formation of the junction.

Redundancy and ligand–receptor interactions that mediate parasite adhesion during erythrocyte invasion

Plasmodium merozoites have evolved a large repertoire of parasite ligands that mediate erythrocyte invasion by mediating interactions with erythrocyte receptors during the different steps of invasion (Table 3.1). This redundancy in ligand–receptor interactions is most evident in *P. falciparum* that can

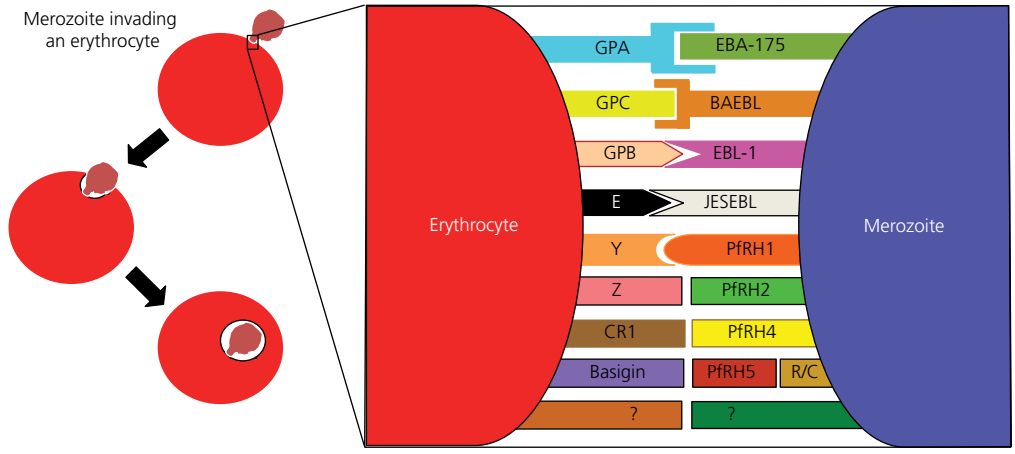


Figure 3.9 EBA/PfRH Ligand-receptor interactions involved in erythrocyte invasion by *P. falciparum*. Schematic representation of the interactions between the *P. falciparum* EBA and PfRH ligand proteins, with their corresponding erythrocyte receptors that mediate erythrocyte invasion. These molecular interactions are responsible for imparting redundancy to the erythrocyte invasion process. The erythrocyte receptors for some parasite ligands still remain unknown and are thus referred in literature by the letters "E", "Y", and "Z". "?" refers to the unknown ligands and receptors that have yet not been discovered. PfRH5 is tethered by Ripr and CyRPA denoted as "R/C".

invade erythrocytes through a number of alternative pathways. In the case of *P. vivax*, the central dogma, as enumerated in the following section, has been that the parasite is solely dependent on the interaction of the *P. vivax* Duffy binding protein (PvDBP) with the Duffy antigen receptor for chemokines (DARC) because the parasite fails to invade Duffy-negative erythrocytes. However, with a number of studies reporting infections of *P. vivax* malaria in Duffy-negative individuals, it appears that *P. vivax* has also evolved alternative invasion pathways.

In the case of *P. falciparum*, evidence for redundancy has emerged from the fact that there is no human erythrocyte variant known that is totally refractory to invasion, as has been demonstrated for *P. vivax* and Duffy-negative erythrocytes. Proof for the presence of alternative pathways came initially from the use of enzymatically treated erythrocytes for invasion assays with different *P. falciparum* strains. Use of erythrocytes treated with enzymes such as neuraminidase, which removes sialic acid residues, for invasion assays allowed classification of parasite strains as being sialic acid dependent or independent for invasion. These studies demonstrated that the two types of parasites used distinct receptors and pathways for invading the erythrocyte, a clear indication of redundancy in the invasion process. Other enzymes that have been used in such studies are trypsin and chymotrypsin. One of the first *P. falciparum* invasion ligands to be discovered was a homologue of PvDBP, the 175-kD erythrocyte binding antigen (EBA-175) which binds sialic acids on glycophorin A. However, *P. falciparum* can invade glycophorin A–deficient erythrocytes, and the gene encoding EBA-175 can be disrupted without affecting parasite growth. A number of parasite molecules that contribute to redundancy in the invasion process (Figure 3.9) have been identified and found to belong to two major families of proteins (DBL, RBL).

Duffy binding–like family

Much of our earlier understanding of the molecular basis of erythrocyte invasion by *P. falciparum* emanated from analogous studies in *P. vivax* and *P. knowlesi*. Both *P. vivax* and *P. knowlesi* invade human erythrocytes using the Duffy blood group antigen as the receptor. This was inferred based on the observations made by Louis Miller that human erythrocytes that lack the Duffy antigen are

refractory to invasion by *P. vivax and P. knowlesi*. The Duffy antigen is a chemokine receptor (DARC) that binds a family of chemokines including IL-8 and MGSA. The human Duffy blood group system is of great clinical significance and is a major example of the relationship between human genetics and malaria, which is discussed in greater detail in Chapter 17.

In 1975, Miller and coworkers reported that when *P. knowlesi* merozoites interact with Duffy-negative human erythrocytes, initial attachment and apical reorientation occur normally, but junction formation does not occur and invasion is aborted (Dvorak 1975). The discovery of the Duffy antigen being a crucial receptor for *P. vivax* and *P. knowlesi* invasion of human erythrocytes led to the identification of *P. vivax* Duffy binding protein (PvDBP) and the *P. knowlesi* Duffy binding protein (PkDBP), the parasite ligands that bind with the Duffy antigen. The 140-kDa PvDBP protein and a 135-kDa PkDBP protein, which specifically bind only Duffy-positive erythrocytes and not Duffy- negative erythrocytes, were identified. Conversely, deletion of the gene encoding PkDBP also renders *P. knowlesi* merozoites unable to form a junction with and invade Duffy-positive human erythrocytes (Singh 2005). These studies demonstrate that the PkDBP–DARC interaction plays a critical role in junction formation during invasion, although the studies do not indicate whether PkDBP is localized at the junction during invasion.

Duffy binding proteins (PvDBP and PkDBP)

The interaction of *P. vivax* and *P. knowlesi* with DARC is mediated by conserved N-terminal cysteine-rich domains within PvDBP and PkDBP that are referred to as region II (PvDBPII and PkDBPII). While PvDBPII specifically binds the human Duffy blood group antigen, PkDBPII binds both the human and rhesus Duffy blood group antigens. Homologous cysteine-rich domains, which are now referred to as Duffy binding–like (DBL) domains, are found on the *P. falciparum* erythrocyte-binding proteins EBA-175, EBA-140, and EBA-181 as well as the variant surface antigens of *P. falciparum* that are encoded by *var* genes and mediate cytoadherence. DBL domains contain around 330 amino acids, including conserved cysteines that form structurally important disulfide linkages and conserved hydrophobic amino acid residues that make hydrophobic interactions, respectively. The binding site on DARC has been mapped to its N-terminal extracellular region (Chitnis 1996). A critical element of the binding site on DARC for PvDBPII incudes a sulfated tyrosine at position 41 (Choe 2005; Hans 2005). Indeed, a second gene encoding a protein homologous to PvDBP has been identified (Menard 2013). The binding specificity of the PvDBP homologue is yet to be determined.

The crystal structures of PkDBPII and PvDBPII have been solved. Both PkDBPII and PvDBPII contain 12 conserved cysteines and are divided into three subdomains with intradomain disulfide bonds (Singh 2006; Batchelor 2011). The three subdomains interact through hydrophobic interactions, which hold them together. Site-directed mutagenesis of PvDBPII, together with functional binding assays, identified amino acid residues in the central 170 amino acid regions that make contact with DARC (Hans 2005). These residues, which lie within subdomain 2, are discontinuous but come together on the surface to form a relatively flat, surface-exposed binding site composed of nonpolar residues (Tyr 94, Leu 168 and Ile 175) as well as polar residues (Asn 95, Lys 96, and Arg 103). Importantly, a majority of the polymorphic amino acids reported in *P. vivax* field isolates from Papua New Guinea and Colombia (Cole-Tobian and King 2003; Tsuboi 1994; Xainli 2000; Ampudia, 1996) are distal to the DARC binding site in subdomain 2. Given that the DARC recognition site appears to be conserved, inhibitory antibodies raised against PvDBPII should be effective against diverse *P. vivax* strains. The overall structure of PkDBPII is similar to the F1/F2 DBL domains from *P. falciparum* EBA-175 (Tolia 2005). A receptor-mediated dimerization model has been proposed for PvDBPII (Batchelor 2011).

It is possible that there are other residues involved in binding of PvDBPII to DARC in additional or overlapping binding regions (VanBuskirk 2004; Chootong 2010; Batchelor 2011; Siddiqui 2012).

However, the residues and recognition site identified (Singh 2006) appear to be an important part of the binding site, as supported by a recent glycan masking study of PvDBPII (Sampath 2013). N-glycosylation at a site adjacent to the predicted DARC interaction site in subdomain 2 abolished PvDBPII interaction with DARC and resulted in weaker inhibitory antibody responses when the glycosylated PvDBPII was used for immunization (Sampath 2013). In contrast, addition of N-glycosylation sites distant from the predicted DARC-binding site or at the predicted dimer interface did not inhibit binding, and they elicited better PvDBPII–DARC binding inhibitory antibodies when compared to wild-type PvDBPII (Sampath 2013). These findings substantiate the role of subdomain 2 interaction site of PvDBPII in binding to DARC.

P. knowlesi also has two other homologous genes that encode the β and γ proteins, which bind rhesus erythrocytes. Region II of the β and γ proteins specifically binds rhesus erythrocytes. Moreover, region II of the β and γ proteins binds chymotrypsin treated rhesus erythrocytes, which have lost the Duffy blood group antigen. *P. knowlesi* is known to invade both normal and chymotrypsin-treated rhesus erythrocytes, suggesting that *P. knowlesi* exhibits redundancy in its invasion of rhesus erythrocytes. Thus, *P. knowlesi* is not solely dependent on the Duffy blood group antigen for rhesus erythrocyte invasion and has the ability to use alternative pathways that may be mediated by the β and γ proteins.

P. falciparum erythrocyte-binding antigens (EBAs)

P. falciparum has a number of homologues of PvDBP known as erythrocyte binding antigens (EBAs) with characteristic cysteine-rich DBL domains that bind different erythrocyte receptors (Adams 2001; Gaur 2004; Iyer 2007; Gaur and Chitnis 2011; Cowman 2012) (Figure 3.10). Unlike *P. vivax*, *P. falciparum* is not dependent on the Duffy antigen for erythrocyte invasion, and the EBAs are known to primarily bind with sialoglycoproteins on the erythrocyte surface, the glycophorins. There are five EBA proteins that are named primarily on the basis of their molecular size: EBA-175, EBA-140 (BAEBL), EBA-181 (JESEBL), EBL-1, and EBA-165 (PEBL), which is a pseudogene that does not translate into a functional protein. The different EBAs are described in detail below.

Erythrocyte binding antigen 175

Among the *P. falciparum* parasite ligands, EBA-175 was the first to be reported (Camus and Hadley 1985). It was discovered in *P. falciparum* culture supernatants and reported to exhibit erythrocyte binding activity, which was dependent on sialic acids. It was later reported to specifically bind with sialic acids on glycophorin A (Orlandi 1992). Another sialoglycoprotein on the erythrocyte surface, glycophorin B, contains identical 11 O-linked oligosaccharides as found in glycophorin A, but it failed to bind with EBA-175, implying that apart from the sialic acid moieties, the peptide backbone of the erythrocyte receptor also has a crucial role to play in the binding of EBA-175 (Dolan 1994). Like the Duffy binding protein of *P. vivax* and *P. knowlesi*, the erythrocyte binding proteins contain conserved cysteine-rich domains, in which cysteine residues are strictly conserved along with the majority of aromatic amino acid residues, suggesting a conserved structure (Adams 1992; Adams 2001). However, there is significant sequence diversity among members of the erythrocyte binding protein family, which likely provides them different binding specificities.

The receptor-binding domain of EBA-175 maps to the N-terminal cysteine-rich region, region II (EBA175RII), which contains duplicated DBL domains in tandem referred to as F1 and F2 (Sim 1994) (Figure 3.10). EBA175RII exhibits limited polymorphisms, especially in its RII receptor binding region. However, these polymorphisms have no effect on receptor specificity, and the different EBA175RII variants always binds in a sialic acid–dependent manner (Jiang 2011). Antibodies raised against the recombinant EBA175 RII protein from *P. falciparum* 3D7 inhibits the

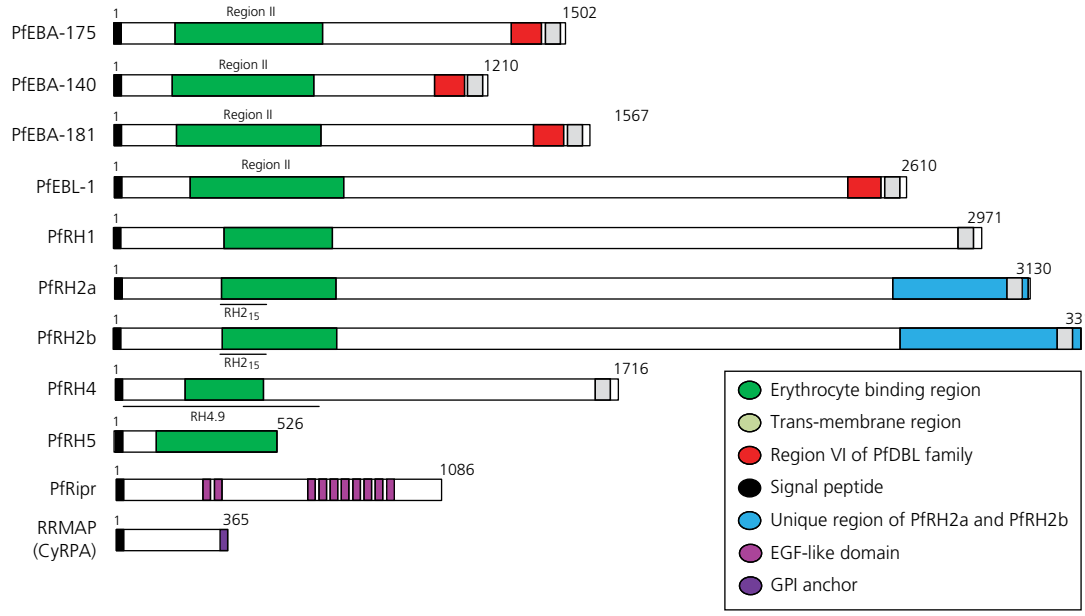


Figure 3.10 The structural features of the EBA and PfRH family of proteins are depicted. The erythrocyte-binding domains of the ligand proteins are shaded in green, which among the EBA proteins are known as Region II. The *lines* in PfRH2 and PfRH4 also represent regions that have been reported to bind erythrocytes. The color-coded structural features of the proteins are shown in the key. PfRipr and RRMAP (CyRPA) form a multiprotein complex with PfRH5 that is the unique PfRH protein lacking a transmembrane domain. PfRipr comprises 10 EGF domains, and RRMAP (CyRPA) is GPI-anchored.

binding of native EBA-175 from three other heterologous *P. falciparum* strains (Jiang 2011). Further, antibodies raised against 3D7 EBA175RII displayed similar erythrocyte invasion inhibitory activity against the three heterologous *P. falciparum* strains (Jiang 2011). Due to its sialic binding phenotype, it was earlier believed that EBA-175 would primarily be used only by the sialic acid–dependent *P. falciparum* clones. However, it has been demonstrated that *P. falciparum* field isolates with diverse invasion phenotypes use the EBA175-glycoporin A pathway for invasion (Badiane 2013). Importantly, antibodies against EBA175RII cross-react to block invasion by diverse *P. falciparum* strains, providing support for inclusion of EBA175 in a receptor-blocking blood-stage malaria vaccine (Duraisingh 2003a; Jiang 2011).

The structural basis for the EBA-175 glycophorin A pathway was elucidated when the crystal structure of EBA175RII was reported at a resolution of 2.3 Å (Tolia 2005). The structure revealed EBA175RII forms dimers with a large number of interactions between the two elongated monomers and two distinct channels at the dimer interface. The F1 and F2 domains are mostly helical and have very similar structures. In the crystal structure, two symmetrical RII molecules interact with each other in an antiparallel handshake manner, with the F1 domain of one monomer interacting with the F2 domains of the second monomer. Cocrystallization of RII with the glycan α-2,3-sialyllactose revealed six glycan binding sites in the RII dimer, all located at the dimer interface. All six glycans made contacts with both monomers, suggesting that dimerization was critical for receptor binding. Based on the structure of the RII–sialyllactose complex, a functional analysis was performed in which the glycan binding sites were mutated and were observed to disrupt the binding of RII with sialyllactose. This study inferred that all six binding sites are important for binding of RII with glycophorin A.

Glycophorin A also exists as a transmembrane dimer with two heavily glycosylated extracellular domains per dimer. These extracellular domains have been reported to inhibit the binding of glycophorin A with EBA175RII, thus suggesting that it is this region that acts as the receptor for EBA175RII. The crystal structure of the RII–sialyllactose suggests that the RII dimer assembles around the dimeric glycophorin A domains and that the dimerization of EBA175RII may be triggered by receptor binding. This is consistent with several observations where EBA175RII has been predominantly found to be in a stable monomeric state in solution.

The x-ray crystal structure data have also helped in understanding the structural basis of invasion inhibition by the EBA-175 antibodies. Structures of the EBA175RII complex with a potent invasion inhibitory monoclonal antibody R217 has shown that the antibody binds with critical residues that are involved in binding with glycophorin A as well as the RII dimer interface (Ambroggio 2013; Chen 2013). Thus, R217 abrogates ligand–receptor interaction, leading to a blockade of erythrocyte invasion. On the other hand, a weak inhibitory antibody R218 was found to bind to an asparagine-rich surface loop and had no effect on receptor engagement, consistent with its poor invasion inhibitory activity (Ambroggio 2013; Chen 2013).

Although to date the focus of structure–function studies of EBA-175 have been on the region II receptor binding domain, a few reports have demonstrated a significant role for the adjoining region III–V, which induces highly potent invasion inhibitory antibodies (Lopaticki 2011; Healer 2013). Thus, further studies are needed to understand the structural and functional basis of the EBA175RIII–V invasion inhibitory antibodies.

Although EBA-175 plays an important role in merozoite invasion of erythrocytes, it was reported that two *P. falciparum* clones with partially disrupted forms of EBA-175 invaded the erythrocytes independently of the glycophorin A pathway (Kaneko 2000; Duraisingh 2003a). This suggested that *P. falciparum* is able to use alternative pathways for invasion. Further evidence for the presence of such alternative pathways was provided by strains of *P. falciparum* that bind to glycophorin A–deficient erythrocytes and sialic acid–deficient erythrocytes (Dolan 1994; Okoyeh 1999). *P. falciparum* parasites were demonstrated to have the ability to switch from sialic acid–dependent to sialic acid–independent invasion phenotypes (Stubbs 2005; Gaur 2006). The sialic acid–dependent *P. falciparum* strain Dd2, when cultured under selection pressure with sialic acid–depleted (neuraminidase-treated) erythrocytes, led to the selection of parasites, denoted Dd2/Nm, that had switched their invasion phenotypes and were able to invade in a sialic acid–independent manner (Gaur 2006). Such mechanisms enable the parasite to invade erythrocytes through alternative pathways. The completion of the *P. falciparum* genome revealed a number of orthologues of EBA-175, which were all located in the sub-telomeric regions of different chromosomes, an indication that the family of genes had evolved as a result of gene duplications and chromosomal rearrangements. These EBA orthologues are described below.

Erythrocyte binding antigen 140

EBA-140 (BAEBL) is a 140-kDa erythrocyte binding antigen that was discovered as part of a family of EBA-175 orthologues in *P. falciparum* (Mayer 2001; Thompson 2001). Like all DBL family members, it also has a cysteine-rich receptor binding domain that is composed of two conserved DBL domains, F1 and F2. The erythrocyte binding phenotype of EBA140 from the *P. falciparum* strain Dd2/NM was first reported by the Miller laboratory to be neuraminidase and trypsin sensitive, indicating that like EBA-175, the erythrocyte receptor is a sialoglycoprotein (Mayer 2001). However, BAEBL bound glycophorin A–deficient erythrocytes and thus exhibited a different erythrocyte binding specificity than EBA-175. Further, EBA-140 of the Dd2/NM strain binds weakly with erythrocytes having the Gerbich-negative phenotype, whereas EBA-175 binds normally to such erythrocytes (Mayer 2001). The Gerbich-negative phenotype arises from a deletion of exon 3 in

glycophorin C that leads to a truncated glycophorin C and absence of glycophorin D. This study was the first to demonstrate that glycophorin C/D is the likely erythrocyte receptor that binds to EBA-140, which was later substantiated by other independent reports (Maier 2003; Mayer 2006; Jiang 2009).

It also became apparent that antigenic polymorphisms in EBA-140 observed among different *P. falciparum* strains affected its specificity and avidity of erythrocyte binding (Mayer 2002; Maier 2009; Jiang 2009). This was the first *P. falciparum* molecule for which it was reported that single point mutations could alter its receptor specificity, as observed previously for viruses. This phenomenon was later observed in other *P. falciparum* invasion–related proteins (e.g., EBA-181, PfRH5) (Maier 2003; Hayton 2008; Mayer 2009) as well and further highlights the high level of complexity evolved by the parasite in its invasion machinery. The crystal structure of EBA140RII has also been reported at a resolution of 2.4 Å, which reveals that the protein is monomeric, with no potential contacts leading to oligomerization being observed (Malpede 2013). The receptor binding pocket in EBA140RII is located in a distinct region of the DBL fold compared to that observed for EBA175RII or PvDBPII. This implies that the same DBL fold could have several binding pockets that could bind with a wide variety of erythrocyte receptors (Malpede 2013). This is consistent with the phenomenon of mutations in EBA140RII affecting receptor binding specificity and binding avidity (Mayer 2002; Maier 2009; Jiang 2009).

Erythrocyte binding antigen 181

EBA-181 (JESEBL), as the name suggests, is a 181-kDa protein that is an orthologue of EBA-175 and similarly exhibits erythrocyte binding activity (Gilberger 2003). It is also expressed during the late merozoite stages and is localized in the micronemes. Similar to EBA-140, mutations in EBA181RII have also shown to affect receptor-binding specificity (Mayer 2004; Maier 2009). There are no definitive reports on the identification of the host erythrocyte receptor that binds with EBA-181. However, it has been suggested that band 4.1 is the receptor for EBA181RII. Expression of EBA-181 is crucial for the function of another invasion-related ligand, PfRH2 (*P. falciparum* reticulocyte binding-like homologous protein 2), which is discussed next. The cooperativity between the PfRH and EBA proteins, of which EBA-181 is a stellar example, is discussed later in a separate section.

Erythrocyte binding ligand 1

Erythrocyte binding ligand 1 (EBL-1) is a 300-kDa EBA175 homologue (Peterson 2000) that binds the sialoglycoprotein glycophorin B (Mayer 2009; Li 2012). Glycophorin B is the only glycophorin that is resistant to trypsin treatment of erythrocytes and has been considered the major receptor that mediates the trypsin-resistant pathway (Dolan 1994; Gaur 2003). An analysis of two *P. falciparum* genetic crosses showed that the inheritance of *ebl-1* was observed to strongly confer a selective benefit to particular progeny, which displayed a higher growth rate (Peterson 2000). EBL-1 is not expressed in all *P. falciparum* strains due to a frameshift mutation in several strains (Drummond 2005; Githui 2010). A careful inspection of these clones indicates that most *P. falciparum* clones that are sensitive to trypsin treatment such as 7G8 and MCAMP have a non-functional EBL-1 (Drummond 2005), which is consistent with their inability to invade trypsin-treated erythrocytes.

Reticulocyte binding–like (RBL) family

P. vivax reticulocyte binding proteins (PvRBPs)

P. vivax merozoites primarily invade only Duffy-positive reticulocytes rather than mature erythrocytes. Although the interaction between the PvDBP and DARC is essential for successful invasion of human reticulocytes by *P. vivax* merozoites, it does not account for their preferential invasion of reticulocytes, because DARC is expressed on both reticulocytes and mature erythrocytes.

The restricted host cell preference of *P. vivax* has been attributed to another multigene family of reticulocyte binding proteins that are expressed at the apical pole of the merozoites and are implicated in directly attaching to the reticulocyte surface. These proteins were first discovered by Mary Galinski and John Barnwell in 1992, who reported two genes encoding the PvRBP1 and PvRBP2 proteins in the *P. vivax* Belem strain that specifically bound reticulocytes (Galinski 1992). PvRBP-1 is a 350-kDa transmembrane protein that contains a number of cysteine residues as well as an RGD motif. Phylogenetic analysis has suggested that PvRBP1 is homologous with the *P. falciparum* homologues PfRH1 and PfRH4 (Rayner 2001). PvRBP2 is also a large-molecular-weight protein, and it shares significant homology with PvRBP1 in a short region and has a seven amino acid repeat motif at the C-terminus. The similar binding function and colocalization data have led researchers to believe that both PvRBP1 and PvRBP2 may function in concert and may form a non–covalently associated complex.

After the genome of the *P. vivax* Salvador I strain was completely sequenced and reported in 2008 (Carlton 2008), it was apparent that there were many more PvRBP genes than the two discovered almost a decade earlier. The PvRBP family comprises a total of 11 PvRBP genes, of which there nine full-length and two partial genes (Carlton 2008; Bozdech 2008; Li and Han 2012). Among the full-length genes, two are pseudogenes that are transcribed but not translated. The eleven genes can be classified into two PvRBP-1 (four genes) and PvRBP-2 subgroups (seven genes). The protein sequence of PvRBP-1 from the Belem strain exhibits 99% identity with PvRBP-1a (Sal I), which has a short deleted sequence of 16 amino acids. PvRBP-1 displays limited polymorphisms compared to PvRBP-2. PvRBP-2 of the Belem strain is 87% identical with PvRBP-2c (Sal I), but their domain structures are distinct. A similar high degree of antigenic polymorphisms have been detected in the PvRBP-2 subgroup genes PvRBP2a/2b/2c/2d.

The fact that *P. vivax* has evolved a large family of reticulocyte-binding proteins suggests that, like *P. falciparum*, *P. vivax* might also have developed significant redundancy in invasion pathways. It has been believed that *P. vivax* is completely dependent on the interaction with the Duffy antigen for invasion and lacks redundancy. However, a number of reports have shown that Duffy-negative individuals do contract *P. vivax* infections. These reports further imply that *P. vivax* has also developed redundant invasion pathways that may be mediated by the PvRBP proteins. Consistent with their function, the PvRBPs are believed to select the host reticulocytes for invasion by triggering the junction formation only after their binding with the correct host cell. However, this has yet to be experimentally demonstrated, and further research is warranted on the PvRBP family of proteins. Understanding the binding specificity and functional roles of PvRBPs and PvDBPs will not only help our understanding of the molecular basis of *P. vivax* reticulocyte invasion but also validate their potential as novel targets for the development of a *P. vivax* blood-stage malaria vaccine.

P. falciparum reticulocyte binding–like homologous proteins

During the beginning of the 21st century, a number of studies identified homologues of the *P. vivax* reticulocyte binding proteins in *P. falciparum* that were named *P. falciparum* reticulocyte binding–like homologous (PfRH) proteins. Because *P. falciparum* invades all age erythrocytes and does not exhibit any host cell restriction, these proteins are believed to have evolved to mediate invasion of normal erythrocytes (normocytes). As for the PvRBPs, some of the first reports for PfRH1 and PfRH2a/2b came from John Barnwell's group, who had initially called them normocyte binding proteins (NBP-1, NBP-2). Since then, these proteins have been widely termed reticulocyte binding–like homologous proteins (RH), and we are following the same nomenclature.

Like the DBL family of erythrocyte binding antigens discussed above, the PfRH proteins have also been demonstrated to directly bind erythrocytes and thus act as parasite ligands involved in

attachment of the merozoites with the host erythrocyte (Figures 3.9 and 3.10). There are six PfRH members: PfRH1, PfRH2a, PfRH2b, PfRH3, PfRH4 and PfRH5, of which PfRH3 is a pseudogene that does not encode a functional protein; this will not be discussed further in this chapter (Figure 3.10). PfRH2 has undergone gene duplication resulting in two identical genes with short unique C-terminal ends located contiguously on the same chromosome 13.

The PfRH proteins have emerged as key determinants of erythrocyte invasion among a large repertoire of invasion molecules and have thus attracted immense attention in the field. As described below, a number of genome-wide studies such as genetic mapping using a *P. falciparum* cross or transcription profiling using microarray analysis have identified PfRH proteins as the major parasite ligand that mediates the different invasion pathways. In addition, different PfRH proteins have been reported to exhibit a variation in expression among different *P. falciparum* clones that correlates with their invasion phenotype. Further, these proteins exhibit limited polymorphisms, and thus their antibodies do not face the obstacle of immune evasion, as observed for leading vaccine candidates like AMA-1. The details for each PfRH member are described below.

P. falciparum reticulocyte binding–like homologous protein 1

The *P. falciparum* orthologue of PvRBP1, PfRH1 (PfNBP1), was first identified by John Barnwell's group in 2001 (Rayner 2001). PfRH1 was reported to exhibit erythrocyte binding activity and bound trypsin-treated erythrocytes while being sensitive to neuraminidase-treated erythrocytes. Thus, PfRH1 is believed to bind a sialic–acid containing, trypsin-resistant erythrocyte receptor that remains unknown to date and has been named receptor Y (Triglia 2005). The dependence of PfRH1 on sialic acids for erythrocyte binding is consistent with the high expression of the PfRH1 protein in *P. falciparum* strains Dd2, MCAMP, FCR3, and T994, which are known to be completely dependent on sialic acids for erythrocyte invasion (Triglia 2005).

PfRH1 is a 360-kDa type-I transmembrane protein that has a large ectodomain and a short cytoplasmic tail. Its 9.6 kb gene is located on chromosome 4. The PfRH1 gene copy number has been reported to differ between different *P. falciparum* strains. Most sialic acid–dependent lines exhibit a high copy number, as observed for FCR3 (13 copies), W2mef (3 copies), FCB1 (4 copies), whereas T994, 7G8, and MCAMP have a single copy (Triglia 2005). The gene copy numbers correlate with the levels of protein detected among the different *P. falciparum* clones. Targeted disruption of the PfRH1 gene in the sialic acid–dependent parasite clones produced knockout parasites that exhibited an increased efficiency to invade both neuraminidase-treated and trypsin-treated erythrocytes.

This increased invasion ability suggests that the knockout parasites no longer use sialic acids for invasion but instead use erythrocyte receptors that are neuraminidase and tryspin resistant. Thus, the deletion of the PfRH1 gene has forced the parasite to compensate by invading through sialic acid–independent pathways and suggests that PfRH1 is the key ligand for sialic acid–dependent invasion.

The large 2937–amino acid PfRH1 protein has been shown to undergo proteolytic processing, which has been consistently reported for a large number of *P. falciparum* invasion proteins such as MSP-1 and AMA-1. PfRH1 is processed post-Golgi into a 240-kDa N-terminal fragment and a 120-kDa fragment containing the transmembrane region. The two processed fragments do not appear to form a complex together because antibodies against one fragment failed to co-immunoprecipitate the other fragment, and thus it is speculated that the 240-kDa PfRH1 fragment may be escorted by another transmembrane protein to the merozoite surface during invasion (Triglia 2009). Both of the two processed fragments were found to colocalize with the moving junction marker RON4, indicating that PfRH1 constitutes the moving junction. The 240-kDa fragment undergoes further processing to a 140-kDa fragment that gets shed into the culture supernatant and also exhibits

erythrocyte binding activity. On the other hand, the 120-kDa fragment comprising the transmembrane region also undergoes processing to yield a 110-kDa fragment in the supernatant. This cleavage is consistent with the action of the *P. falciparum* rhomboid proteases that are known to cleave proteins in their transmembrane helices. *P. falciparum* rhomboid proteases have been shown to cleave a number of parasite ligands including the PfRH proteins. The physiological significance of this cleavage is that it allows disengagement of the ligand–receptor interactions, facilitating parasite movement during invasion.

The large molecular weight PfRH proteins such as PfRH1, PfRH2, and PfRH4 have large ectodomains, and during the last several years a number of studies have mapped the receptor binding domains of these parasite ligands. Unlike the DBL family of proteins that have a conserved cysteine-rich region as a signature in each of its members, the PfRH proteins lack such highly conserved domains. However, they do exhibit homology in their N-terminal regions that have proven to be their receptor binding domains. Whereas the receptor binding domain for PfRH4 was reported first (Gaur 2007), the receptor binding domain of PfRH1 was also mapped to its N-terminal region that shares homology with PfRH4 (Gao 2008) (Figure 3.10). In an elegant study, Peter Preiser's group expressed overlapping regions of the entire ectodomain on the surface of COS cells and observed their ability to bind human erythrocytes and form rosettes. This resetting assay was the basis for the identification of the cysteine rich-DBL region II as the receptor binding domains of PvDBP and PfEBA-175. Through this assay, it was reported that the region of PfRH1 encompassing residues 333 to 1000 exhibited erythrocyte-binding activity. The minimal binding region was further mapped between residues 500 and 833. PfRH1 binds erythrocytes in a sialic acid–dependent, trypsin-resistant manner, and the receptor binding region of PfRH1 binds with the same specificity.

The precise localization of PfRH1 as defined by immunoelectron microscopy has not yet been reported. PfRH proteins have been labeled rhoptry proteins, but this has been proved by immunoelectron microscopy only for PfRH2 and PfRH5. This assumption has led to some conflicting results reported with regard to the calcium signals that induce the sequential release of the apical organelles. It has been demonstrated both in *Toxoplasma* and *Plasmodium* species that increase in intracellular calcium levels triggers release of micronemal proteins, which in turn bind with the erythrocyte and further induces the release of the rhoptries. A study has shown that monoclonal antibodies against PfRH1 that block invasion specifically inhibit calcium signaling and microneme secretion (Gao 2013). In light of the fact that it is yet not clear where PfRH1 is localized, more research is needed to resolve this paradox.

P. falciparum reticulocyte binding–like homologous protein 2

PfRH2a and PfRH2b were the first members of the PfRH family to be identified by the laboratories of John Barnwell and Alan Cowman, and these were followed by the identifications of PfRH1, PfRH4, and PfRH5. PfRH2a and PfRH2b are encoded by two adjacent genes that have high sequence identity, are located in a head-to-head orientation on chromosome 13, and appear to have evolved by gene duplication (Rayner 2000; Duraisingh 2003b). The two 9-kb genes share an identical 8-kb region that encodes for around 2700 amino acids. The difference between the gene sequences arises toward their distinct 3′ ends that comprises around 500 amino acids toward the C-terminus. This unique region comprises a short portion of the ectodomain, the transmembrane region, and the cytoplasmic region. The identical ectodomain region between PfRH2a and PfRH2b, comprising 2700 amino acids, is referred as PfRH2a/b.

Recent interest in the PfRH family of proteins has primarily arisen due to the strong correlation between their expression with the invasion phenotypes of different parasite lines (Triglia 2005). This phenotypic variation of the PfRH proteins has positioned them as key determinants of different invasion pathways (Triglia 2005). The sialic acid–dependent strains, such as Dd2, MCAMP, and

FCR3, express higher levels of PfRH1 and low levels of PfRH2a/2b. On the other hand, the sialic acid–independent strains, such as 3D7, HB3, and 7G8, express higher levels of PfRH2a/2b and lower levels of PfRH1.

Previously, PfRH2a/b were implicated to play a role in erythrocyte invasion based on genetic manipulation of the two genes because their erythrocyte binding activity could not be detected (Duraisingh 2003b). The invasion phenotype differences between the PfRH2b knockout and wild-type parasite line suggested that PfRH2b determines erythrocyte invasion through a sialic acid–independent, trypsin-resistant, chymotrypsin-sensitive pathway. Among the two proteins, PfRH2b is believed to play a more dominant and important role, while PfRH2a has more of a secondary accessory function. This is exemplified in a report that showed that only the cytoplasmic tail of PfRH2b bound aldolase, whereas the PfRH2a tail failed to show a similar aldolase-binding property (Pal-Bhowmick 2012).

A number of studies have demonstrated that proteolytically processed fragments of PfRH2a and PfRH2b exhibit distinct erythrocyte binding activity (Sahar 2011; Triglia 2011). Proteolytic processing has been well reported for a number of invasion-related parasite proteins such as MSP-1, AMA-1, PfRH1, PfRH4, and PfRH5. Proteolytic processing of parasite ligands during invasion is catalyzed by a number of specific proteases such as rhomboids and subtilisins. The large-molecular-weight 350-kDa PfRH2 protein has been observed to undergo a two-step processing that yields fragments of 220 to 270 kDa, 140 kDa and 80 kDa (Gunalan 2011; Sahar 2011).

Two studies have reported binding of a large 220- to 270-kDa PfRH2 fragment to erythrocytes in a sialic acid–independent manner consistent with previous reports (Gunalan 2011; Sahar 2011). In addition, a smaller 80-kDa processed PfRH2 fragment binds erythrocytes in a sialic acid–dependent manner. Whereas the erythrocyte binding phenotype of the larger fragment is consistent with the role of PfRH2 in erythrocyte invasion by a sialic acid–independent pathway, the physiological significance of the binding of the 80-kDa processed fragment in the invasion process is unclear. These reports clearly demonstrate that differentially processed fragments of the same *P. falciparum* protein may bind with different erythrocyte receptors, further implying another level of complexity within the parasite's invasion process.

These observations for PfRH2 are similar to that reported for the erythrocyte binding of EBA-175, which has been shown to bind glycophorin A in a sialic acid–dependent manner. The full-length EBA-175 protein fragment of around 150 to 160 kDa binds erythrocytes, and its receptor binding domain (region II) has also been well characterized. However, in addition it has also been reported that a 65-kDa processed fragment of EBA-175 exhibits erythrocyte binding in a sialic acid–independent manner. Thus while EBA-175/glycophorin A interaction mediates invasion through the sialic acid–dependent pathway, the physiological significance of the binding of the 65-kDa fragment remains unclear to date (Duraisingh 2003a).

The erythrocyte binding domain of PfRH2 has also been mapped to a 40-kDa region ($rPfRH2_{40}$, amino acids 495–860) near its N-terminal end consistent with that observed for other PfRH members (Sahar 2011). Recombinant $PfRH2_{40}$ binds erythrocytes with the same specificity as the native 220-kDa PfRH2a/b protein and is consistent with the role of PfRH2a/b in the sialic acid–independent, trypsin-resistant, and chymotrypsin-sensitive invasion pathway. Another study has shown that a 15-kDa region that overlaps with the 40-kDa region also exhibits erythrocyte binding, further confirming this region as the receptor binding domain (Triglia 2011). The elucidation of the erythrocyte binding domain of PfRH2 is also significant because antibodies raised against this domain exhibit potent invasion inhibitory activity.

The N-terminal region of PfRH2 has more polymorphisms compared to the rest of the protein and is considered a target of protective immunity against *P. falciparum* malaria. Analysis of 33 PfRH2 sequences showed a region (amino acids 212–745) encompassing the receptor binding domain was

most polymorphic and suggested that it was under diversifying selection due to immune pressure (Reiling 2010). It was also observed that human antibodies against this polymorphic N-terminal region of PfRH2 were strongly associated with protection against high-density parasitemia. The polymorphic N-terminal region of PfRH2 has a significant overlap with the $rPfRH2_{40}$ receptor-binding region (amino acids 495–860). Protein domains involved in engagement with the host erythrocyte would be naturally exposed to the human immune system and therefore be under immune pressure. Hence, these data further confirm that the N-terminal region of PfRH2 constitutes its functional erythrocyte binding domain.

P. falciparum reticulocyte binding–like homologous protein 4

PfRH4 was the first member of the PfRH family whose functional significance highlighted the PfRH proteins as key determinants of erythrocyte invasion (Stubbs 2005; Gaur 2006). PfRH4 is a 220-kDa transmembrane protein that is smaller than both PfRH1 and PfRH2. PfRH4 was first identified as the effector parasite molecule involved in the sialic acid–independent pathway used by *P. falciparum* for erythrocyte invasion. Two independent reports reported that upregulation in expression of two adjacent genes (PfRH4 and PfEBA-165/PEBL) on chromosome 4 was associated with a switch in the invasion phenotype of the *P. falciparum* clone Dd2 from sialic acid dependence to independence (Dd2/NM) (Stubbs 2005; Gaur 2006).

In 1990, Stephen Dolan along with Tom Wellems and Louis Miller had first reported that the *P. falciparum* clone Dd2, which is completely dependent on sialic acid–containing glycoproteins on the erythrocyte surface for invasion, has the ability to switch its invasion phenotype when cultured under selection pressure in the presence of sialic acid–depleted (neuraminidase treated) erythrocytes (Dolan 1990). This interesting observation suggested that the parasite genome exhibits plasticity to counter different changes in its environment. Because Dd2/NM is a selected subclone of the parental Dd2 strain, both parasites have an identical genetic background, and thus gene expression profiling at the level of RNA transcription demonstrated that the expression of genes encoding PfRH4 and PEBL (EBA-165) was found to be upregulated in Dd2/Nm compared to Dd2. PEBL or EBA-165 is a pseudogene that does not encode a functional parasite protein. As a result, PfRH4 appeared to be the major effector molecule that was implicated in sialic acid–independent invasion. This was confirmed when it was demonstrated that deletion of PfRH4 in Dd2 results in parasites that fail to switch their invasion phenotype.

Further biochemical characterization demonstrated that PfRH4 exhibits erythrocyte binding activity in a sialic acid–independent manner consistent with their upregulation in Dd2/NM (Gaur 2007; Tham 2009). PfRH4 was the first RBL member whose receptor-binding domain was mapped to a conserved region near the N-terminus of the native protein (Gaur 2007; Tham 2009). Later, complement receptor-1 (CR1) was identified as the erythrocyte receptor for PfRH4, and minimal binding regions have been mapped in both PfRH4 and CR1 (Tham 2010; Tham 2011). Although the PfRH4-CR1 molecular interaction appears to mediate a major sialic acid–independent invasion pathway, antibodies raised against PfRH4 do not exhibit potent invasion inhibitory activity individually but do produce additive or synergistic inhibition in combination with antibodies against other merozoite antigens (Williams 2012; Pandey 2013).

P. falciparum reticulocyte binding–like homologous protein 5

PfRH5 is a unique member of the PfRH family due to its essential functional role in merozoite invasion because unlike other members, its gene cannot be disrupted by genetic manipulation (Baum 2009). It was first discovered through the study of a *P. falciparum* genetic cross as a key determinant of species-specific erythrocyte invasion (Hayton 2008). The analysis of the invasion

phenotypes of 33 recombinant progeny clones from a cross between two *P. falciparum* strains 7G8 × GB4 along with their genotypes led to the identification of the PfRH5 gene as the key mediator of invasion of *Aotus nancymaae* erythrocytes as well as infectivity of *Aotus* monkeys. Like other parasite ligands, it was also demonstrated that PfRH5 is a parasite adhesin that bound human erythrocytes and in which single point mutations critically influenced its specificity of binding *Aotus* erythrocytes. On similar lines, PfRH5 plays an important role in rat erythrocyte invasion by *P. falciparum* (Hayton 2013).

Whereas the PfRH proteins (PfRH1, PfRH2, PfRH4) are differentially expressed among different *P. falciparum* clones that exhibit phenotypic variation in their invasion properties, PfRH5 was observed to be consistently expressed among these parasite clones, which further substantiates its critical role in *P. falciparum* erythrocyte invasion (Baum 2009).

The CD147 IgG super family member basigin (BSG) was discovered to be the erythrocyte receptor for PfRH5 (Crosnier 2011). This important discovery was made through the AVEXIS (Crosnier 2011) approach using a library of erythrocyte surface receptors that were expressed in mammalian cells and tested for binding to PfRH5 expressed as a multimeric fusion protein to increase avidity. The physiological significance of the PfRH5-BSG molecular interaction was accentuated by the observation that anti-BSG antibodies targeting a single receptor or molecular interaction potently blocked erythrocyte invasion of multiple *P. falciparum* clones. The broad strain-transcending invasion inhibitory activity of the anti-BSG antibodies substantiated the conserved critical role of the PfRH5-BSG interaction for erythrocyte invasion and projected PfRH5 as an attractive vaccine target.

Following this discovery, a number of studies have reported generation of potent anti-PfRH5 neutralizing antibodies that efficiently blocked *P. falciparum* erythrocyte invasion in a strain-transcending manner. These include the heterologous prime-boost approach to generate anti-PfRH5 neutralizing antibodies using the adenoviral/MVA viral vector platform (Douglas 2011). Similarly, antibodies raised against the PfRH5-ratCD4(d3 + 4) fusion protein exhibit potent inhibition of erythrocyte invasion (Bustamante 2012). Recombinant full-length PfRH5 produced in *E. coli,* which binds erythrocytes with the same specificity of native PfRH5, also elicits potent strain-transcending invasion-inhibitory activity as observed with those antibodies raised through the adenoviral vectors or PfRH5-ratCD4(d3 + 4) fusion protein (Reddy 2014). The production of recombinant wild-type full-length PfRH5, which would elicit similar potent invasion-inhibitory antibodies in a bacterial expression system such as *E. coli* that has a high feasibility for mass production, has huge implications for blood-stage malaria vaccine development.

In another major development that has strong implications for vaccine design, the three-dimensional structure of PfRH5 has been solved by two independent groups (Chen 2014; Wright 2014). Native PfRH5 protein is known to be processed into a smaller 45-kDa fragment that retains erythrocyte binding activity. Similarly, recombinant full-length PfRH5 is also unstable and gets cleaved at its N-terminus to yield a processed 45-kDa fragment. Thus, while one group focused on the 45-kDa PfRH5 fragment (amino acids 127–526) (Chen 2014), the other group first predicted a long disordered region within the residues 1 to 140 and 248 to 596 and solved the structure of a truncated version, PfRH5ΔNL (residues 140–526 lacking region 248–296) (Figure 3.11) (Wright 2014).

Both studies have reported PfRH5 to exhibit a novel fold in which two triplet-helical bundles assemble to form a kite-like structure. The PfRH5 structure consists of two disulfide bonds, with one disulfide bond (Cys_{345}–Cys_{351}) stabilizing the loop that connects the two helical bundles, whereas the other disulfide bond (Cys_{224}–Cys_{317}) connects the second and third helix. Due to polymorphism at amino acid position 203 of PfRH5 (Hayton 2008; Crosnier 2011), the 3D7PfRH5 variant consists of

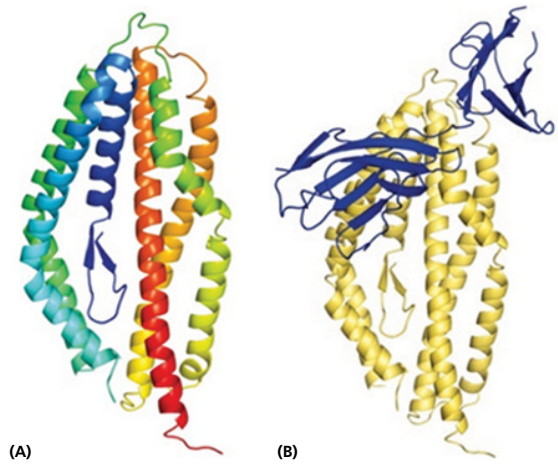


Figure 3.11 Three-dimensional structure of PfRH5 and basigin. *A,* Crystal structure of the PfRH5 protein. *B,* The structure of PfRH5 (depicted in *yellow*) bound to basigin (depicted in *blue*). *Source:* Wright KE, Hjerrild KA, Bartlett J, Douglas AD, Jin J, *et al.* 2014. Structure of malaria invasion protein RH5 with erythrocyte basigin and blocking antibodies. *Nature.* 515(7527):427–430. (*See insert for color representation of this figure*.)

six cysteines (Chen 2014), whereas the 7G8 variant consists of five cysteines (Wright 2014). However in the 3D7 PfRH5 variant, the Cys203 residue is free with partly exposed side chain (Chen 2014). The side chain of the other free cysteine residue at position 329 is completely buried within the structure (Chen 2014; Wright 2014).

Basigin has been reported to act as the receptor for PfRH5 binding on to the erythrocyte surface (Crosnier 2011). Basigin belongs to the immunoglobulin superfamily (IgSF) that exists in two forms, BSG-L (three IgSF domains) and BSG-S (two IgSF domains). Crystal structure of PfRH5ΔNL with BSG-S shows that most of the contact occurs between the basigin N-terminal domain and the loops at the tip of PfRH5 through hydrogen bonds. This interaction is further stabilized by F_{350} and W_{447} residues of PfRH5, which pack into the hydrophobic pockets of basigin. The C-terminal domain and the residue H102 in the linker region of basigin also flexibly interact with the fourth and fifth helix of PfRH5. Limited polymorphisms have been reported for PfRH5, but these polymorphic residues do not directly interact with basigin. However, some polymorphisms that dictate host tropism (Hayton 2008; Hayton 2013; Wanaguru 2013) lie in close proximity to the basigin binding site (Wright 2014).

Previously, PfRH5 monoclonal antibodies have been reported to inhibit parasite growth (Douglas 2013). Two of them, QA1 and QA5, abrogate the PfRH5–BSG interaction. QA1 binding region overlaps with the basigin N-terminal domain, whereas QA5 majorly interacts with the second helix of PfRH5 that overlaps with the basigin C-terminal domain binding region (Wright 2014). In contrast, another inhibitory monoclonal antibody 9AD4 that does not impede PfRH5–BSG interaction binds close to but not coincident with both the basigin binding sites (Wright 2014). This suggests that antibodies targeting the epitopes in and around the basigin binding sites are highly potent.

Another important aspect of PfRH5 is that it exists as a complex of three parasite proteins, PfRH5, PfRipr, and CyRPA (Chen 2011; Reddy 2015), which is discussed below. Reports suggest that antibodies targeting the two interacting proteins PfRipr and CyRPA display potent invasion-inhibitory activity similar to that of PfRH5 (Chiu 2014; Reddy 2015). It would be interesting as well as highly challenging to solve the structure of the PfRH5–PfRipr–CyRPA complex, which would lead to novel

ways to target the formation of this complex that would efficiently impede erythrocyte invasion and probably confer sterile protection against malaria.

PfRH5–PfRipr–RRMAP adhesin complex

PfRH5 is exceptional compared to other PfRH proteins due to its smaller size (63 kDa) and lack of a transmembrane domain. In the absence of a transmembrane domain and any GPI linkage, a major question with PfRH5 is how it is anchored on the merozoite surface such that it binds with basigin on the erythrocyte surface during the process of erythrocyte invasion. In the absence of a transmembrane domain or GPI-anchor, it is highly likely that PfRH5 may be secured on the merozoite surface by associating with other parasite surface proteins. In this regard, it was reported that PfRH5 associates with another novel parasite molecule, PfRipr (*P. falciparum* RH5 interacting protein) (Chen 2011). The full-length PfRipr (PFC1045c) is a 125 kDa protein that comprises 87 cysteine residues, which constitute 10 EGF-like domains. Like many invasion-related parasite proteins, PfRipr undergoes processing close to its central region to yield two fragments of around 65 kDa that both remain associated with the PfRH5 complex. It is localized in the micronemes and is released to the merozoite surface. PfRipr was observed to not bind with erythrocytes directly, but instead its antibodies have been shown to potently inhibit both merozoite attachment and invasion into human erythrocytes. Efforts to genetically disrupt PfRipr have failed, suggesting an essential role, which is consistent with it forming a complex with an essential and key parasite ligand, PfRH5 (Chen 2011).

However, PfRipr also lacks a transmembrane domain and is not predicted to be a GPI-anchored protein, and hence the question as to how PfRH5 is secured on the merozoite surface during invasion remained unresolved till now. Using the approach of co-immunoprecipitation followed by mass spectrometry, the third interacting partner of PfRH5–PfRipr, CyRPA (Cysteine-rich protective antigen), has been identified and has been demonstrated to be GPI anchored (Reddy 2015). This novel parasite protein, CyRPA tethers the PfRH5–PfRipr proteins to the merozoite surface during invasion and is termed RRMAP (RH5-Ripr membrane anchoring protein) (Reddy 2015). Mature RRMAP is a 35-kDa protein that has been localized to the micronemes and has not been observed to undergo proteolytic processing.

Formation of the multiprotein adhesin PfRH5–PfRipr–RRMAP complex was analyzed by ion-exchange chromatography, size-exclusion chromatography, and native gel electrophoresis. Repeated attempts to genetically disrupt the RRMAP gene failed, which is consistent with its essential role of securing two other essential invasion proteins. All three components of the multiprotein complex were demonstrated to be colocalized on the surface of the apical end of the invading merozoite consistent with their role in securing PfRH5 on the merozoite surface and facilitating its interaction with its erythrocyte receptor, basigin (Reddy 2015) (Figure 3.12). Thus, based on their localization in the micronemes, which are believed to be secreted prior to the rhoptries, it appears that PfRipr and RRMAP associate first either in the micronemes itself or on the merozoite surface and form a platform on which the PfRH5 rhoptry protein is anchored. Similar to PfRipr, RRMAP was also not observed to bind with erythrocytes, thus suggesting that the complex disintegrates as soon as PfRH5 engages with basigin.

RRMAP is a highly conserved antigen, and its antibodies display potent strain-transcending invasion inhibition of the magnitude similar to that observed with PfRH5 antibodies (Reddy 2015). The formation of such multiprotein complexes to execute a single function appears to be an immune evasion strategy by the parasite, in which it is able to keep its most crucial proteins hidden from the human immune system by not having to release them earlier or constitutively on the merozoite surface. The PfRH5–PfRipr–RRMAP complex is a potent target not only for potent vaccines but also for novel drugs that could abrogate the formation of the complex and thus impede erythrocyte invasion.

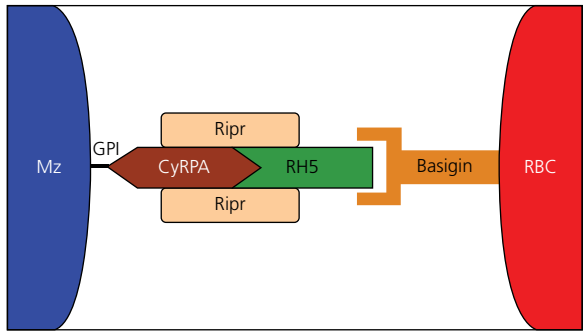


Figure 3.12 The PfRH5-Ripr-CyRPA multiprotein complex is essential for erythrocyte invasion. Whereas the PfRH5–basigin interaction is essential for *P. falciparum* erythrocyte invasion, PfRH5 lacks a transmembrane domain or a GPI-anchor. It has been elucidated that PfRH5 exists on the surface of the invading merozoite as part of a multiprotein complex consisting of Ripr (RH5 interacting protein) and CyRPA (cysteine-rich protective antigen). Ripr is a 125-kDa protein that also lacks a transmembrane domain and is proteolytically processed into two equal fragments of ~65 kDa. CyRPA is the only GPI-anchored protein among the three proteins and is thus the RH5-Ripr membrane anchoring protein.

Cooperativity between the DBL and RBL families of proteins

Several examples have been observed that demonstrate a cooperativity between the two major families of invasion ligands that are responsible for mediating erythrocyte invasion. The first was the observation that the genetic disruption of the EBA-175 gene in the *P. falciparum* clone Dd2 led to upregulation of expression of PfRH4, which turned out to be the switching mechanism that mediated a shift in the invasion preference of the Dd2 clone from a sialic acid–dependent to an independent phenotype. Thus, the loss of a major invasion ligand from the DBL (EBA) family, which would incapacitate the parasite, was compensated for by the significant increase in expression of another PfRH family member. Whereas the genetic manipulation study augurs for cooperativity, the upregulation of PfRH4 expression could occur even in the presence of the EBA-175 gene (i.e., without genetic disruption) under selection pressure.

Another example is related to another DBL–PfRH pair, EBA-181 and PfRH2b (Lopaticki 2011). Interestingly, antibodies against PfRh2b, a major sialic acid–independent ligand that normally exhibits potent invasion inhibition, did not block invasion of parasites lacking EBA-181. The PfRH2b antibodies directed against the C-terminal region of its ectodomain exhibited low invasion inhibition of the parasite lines 3D7Δ181 and 3D7Δ175/181 in comparison to the potent inhibition observed against the 3D7, 3D7Δ175, 3D7Δ140, and 3D7Δ175/140 (Lopaticki 2011). Both 3D7Δ181 and 3D7Δ175/181 lack EBA-181 expression (Lopaticki 2011) but did express the PfRH2b protein. Thus, the low invasion-inhibitory activity of the PfRH2b antibodies could not be accounted for by the lack of expression of the parasite molecule in the EBA-181 knockout parasites. Hence, these data strongly suggest a positive cooperation between PfRh2b and EBA-181 during merozoite invasion. It would be interesting to check whether EBA-181 antibodies would also lack their inhibitory potency against the PfRH2b knockout parasites. A direct physical association between PfRH2b and EBA-181 has not been demonstrated, and this could be due to either the highly transient nature of their interactions, which could be short-lived as well, or the fact that the functional cooperation between these two proteins does not entail a physical contact between themselves. Nevertheless, the cooperative interplay between the DBL (EBA) and PfRH proteins during erythrocyte invasion by *P. falciparum* merozoites has strong implications with regard to their vaccine potential for the development of blood-stage malaria vaccines.

Identification of novel parasite ligands

In addition to the EBA/PfRH proteins, there has been a great expansion in the identification of parasite molecules implicated to play a role in the process of erythrocyte invasion such as PfRipr, RRMAP, AARP, and the rhoptry proteins (RAP, RhopH). PfRipr and RRMAP have been described above in context of PfRH5, and the rest are discussed below.

P. falciparum apical asparagine-rich protein

PfAARP (*P. falciparum* apical asparagine rich protein) was identified as a novel asparagine-rich protein that harbors the predicted features of a surface antigen (signal sequence, transmembrane domain) and whose expression profile matched that of other known invasion-related proteins (Wickramarachchi 2008). It was localized to the apical end of the merozoites by several approaches. Immunoelectron microsopic studies showed that PfAARP is localized in the apical ends of the rhoptries in the merozoites. PfAARP has been demonstrated to exhibit erythrocyte binding activity, which is sensitive to trypsin and neuraminidase treatments, suggesting that PfAARP binds in a sialic acid–dependent manner. The N-terminal region of PfAARP has been observed to be highly conserved among different *P. falciparum* strains as well as highly immunogenic with human immune sera from individuals residing in malaria-endemic areas. The anti-PfAARP antibodies, raised against the N-terminal region that also binds erythrocytes, efficaciously impeded erythrocyte invasion. Antibodies against AARP in combination with other EBA/PfRH antibodies have been shown to produce potent additive or synergistic inhibition (Pandey 2013; Reddy 2014). Thus, PfAARP has been identified as a novel parasite ligand that has strong potential as a blood-stage malaria vaccine candidate.

RhopH complex

The RhopH complex is a high molecular mass multiprotein complex that is expressed at the schizont stage in developing merozoites. It comprises three distinct subunits – RhopH1/clag, RhopH2 and RhopH3 – in an equal stoichiometric ratio of 1:1:1 (Holder 1985; Brown and Coppel 1991; Sam-Yellowe and Perkins 1991; Kaneko 2001; Shirano 2001; Kaneko 2005). It localizes to the rhoptry bulb and gets translocated to the ring stage after invasion in the next cycle (Ling 2004). RhopH1, RhopH2, and RhopH3 have been shown to be secreted and transferred to the parasitophorous vacuole membrane upon erythrocyte invasion. The RhopH complex is also transferred to defined domains of the erythrocyte cytoplasm, and possibly is transiently associated with Maurer's clefts. The RhopH complex was the first *P. falciparum* rhoptry protein demonstrated to be secreted and trafficked in the host erythrocyte upon merozoite invasion.

The RhopH complex is implicated to play a role in both erythrocyte invasion and the subsequent remodeling of the host erythrocyte. RhopH proteins exhibit erythrocyte binding activity, and anti-RhopH antibodies show invasion-inhibitory activity, substantiating their role in erythrocyte invasion (Sam-Yellowe and Perkins 1991; Doury 1994; Wang 2006).

P. falciparum RhopH1 is encoded by either clag2 (PFB0935w), clag3.1 (PFC0120w), clag3.2 (PFC0110w), clag8 (MAL7P1.229), or clag9 (PFI1730w), members of the previously named *clag* (cytoadherence-linked asexual gene) multigene family. The five *rhoph1/clag* gene members were transcribed during the late schizont stage, but these genes exhibit a differential expression. All *rhoph1/clag* member genes were transcribed in HB3 and 3D7 parasite lines, but *clag3.2* was not transcribed in Dd2 parasites. RhopH1/clag has been shown to be encoded by at least three RhopH1/clag members to date: clag2, clag3.1, and clag9 (Ling 2004; Kaneko 2005). Based on their identity in protein structure and expression pattern, clag3.2 and clag8 are also likely to be part of the RhopH complex. The PfRhopH complex comprises one each of RhopH1/clag (155 kDa), RhopH2 (140 kDa),

and RhopH3 (110 kDa) as the estimated total molecular mass of the PfRhopH complex is approximately 480 kDa (Etzion 1991). The PfRhopH complex immunoprecipitated with the anti-clag9 antibody contained neither clag2 nor clag3.1, confirming that the PfRhopH complex consists of only one of the five *rhoph1/clag* encoded proteins. Because RhopH2 and RhopH3 are encoded by single genes and based on the stoichiometry described above; there are at least five different types of PfRhopH complexes expected in the parasite, which may serve to confer some degree of specificity to the roles of the individual complexes.

Signaling events during erythrocyte invasion by malaria parasites

As described above, invasion of erythrocytes is a multistep process that is mediated by multiple molecular interactions between parasite ligands and host receptors. Malaria parasites have evolved significant redundancy at different steps of invasion, with different members of parasite protein families such as the EBA, RBP and RH families capable of interacting with diverse erythrocyte receptors. Surprisingly, many of the parasite ligands that interact with invasion receptors are not located on the merozoite surface but are found in apical organelles referred to as micronemes and rhoptries. For example, AMA1 and members of the EBA family are localized in micronemes, whereas members of the PfRH family, such as PfRH2 and PfRH5, have been localized to the rhoptries.

The sequence and signals that trigger the release of these parasite ligands to the merozoite surface during erythrocyte invasion were not known. It has been demonstrated that exposure of merozoites to a low K^+ environment, as found in blood plasma, provides the signal that leads to a rise in cytosolic cAMP and Ca^{2+}, which triggers release of microneme proteins such as AMA1 and EBA175 to the merozoite surface. When merozoites are exposed to a low-K^+ environment as found in blood plasma, the cytosolic carbonic anhydrase produces bicarbonate (HCO_3^-) ions and protons (H^+) to balance pH. Rise in cytosolic levels of HCO_3^- ions activates the bicarbonate-sensitive soluble adenylyl cyclase (ACβ), leading to production of cAMP. Rise in cytosolic cAMP activates protein kinase A (PKA), which plays a direct role in microneme secretion, and GTP exchange protein activated by cAMP (EPAC). Activated EPAC transfers a GTP to Rap GTPase, leading to its activation. Activated Rap GTPase stimulates phospholipase C (PLC) to produce inositol triphosphate (IP_3) and diacyglycerol (DAG) from phosphatidyl inositol biphosphate (PIP_2). Production of IP_3 triggers release of Ca^{2+} from intracellular Ca^{2+} stores. Rise in cytosolic Ca^{2+} activates calcium-dependent protein kinase 1 (PfCDPK1) and the calcium-dependent phosphatase, calcineurin (PfCN), which have both been shown to play a role in microneme secretion.

Previous studies have also demonstrated a role for the cGMP-dependent protein kinase, PfPKG, in triggering release of proteins from micronemes and exonemes in merozoites in mature schizonts (Blackman 2013). Secretion of exoneme proteins such as the protease PfSub1 plays a critical role in merozoite egress from infected erythrocytes. The external or internal signals that trigger a rise in cGMP and activate PfPKG, leading to egress, are not known. The pathways for cross-talk between cGMP and Ca^{2+} in *P. falciparum* merozoites during egress have not been defined, and whether cGMP-dependent PfPKG plays a role in microneme secretion in free merozoites after egress is not known. It will be important to understand the cross-talk between cGMP, cAMP, and Ca^{2+} in merozoites leading to apical organelle exocytosis both before and after egress.

Interestingly, the rise in cAMP and Ca^{2+} only triggers microneme secretion and not rhoptry secretion. Binding of translocated EBA175 to its erythrocyte receptor, glycophorin A (glyA), restores basal Ca^{2+} levels and triggers release of rhoptry proteins such as PfRH2, clag3.1, and PfTRAMP. The secretion of apical organelles from *P. falciparum* merozoites is thus a sequential process in which

microneme secretion precedes rhoptry secretion. Identification of some of the players involved in the signaling pathways that lead to microneme and rhoptry secretion enabling interactions with host receptors that mediate invasion is not only important for understanding the invasion process but also provides novel targets for drug design. Inhibitors that target the signaling pathways involved in microneme or rhoptry release can block erythrocyte invasion by *Plasmodium* merozoites to limit parasite growth.

Summary and conclusions

Erythrocyte invasion by *Plasmodium* merozoites is a fascinating biological process that has attracted immense attention in the malaria field. As our understanding of this intricate process has improved, it has also highlighted the enormous complexity in the underlying molecular mechanisms that mediate this process. As dictated by the large genome of which 2700 genes are predicted to be expressed at the blood stages, most research in the past three to four decades and also continuing now has focused on a linear expansion of parasite molecules and their functional characterization. This is an important exercise considering that the parasite has evolved a complex molecular machinery with significant redundancy in order to ensure that it can successfully invade diverse host erythrocytes.

It is also clear that parasite molecules do not act in isolation but participate in a complex multistep process leading to host cell invasion. Thus, it is crucial to understand the cross-talk and interaction between the different parasite proteins during the process of erythrocyte invasion. This is exemplified from the discoveries of multiprotein complexes such as PfRH5-PfRipr-RRMAP, MSP1/6/7-MSPDBL, and AMA1-RON that are essential for *P. falciparum* erythrocyte invasion. In fact, in the latter case, the parasite inserts its own molecules, the RON proteins, into the erythrocyte membrane that serves as a receptor for AMA-1. Such a scenario might exist for many other parasite proteins that do not exhibit a direct erythrocyte binding function with human erythrocytes.

Since the turn of the century, the malaria research field has made a gigantic leap in our understanding of the biology of the blood-stage parasites primarily due to the availability of the *P. falciparum* genome and access to advanced technology platforms as well as the advent of innovative methodologies enabling global approaches to study the parasites. However, many questions still remain to be answered before we can get a complete understanding of the molecular basis of erythrocyte invasion. Ultimately, we hope that this will help in identifying the Achilles heel of the parasite, which could be harnessed for the development of successful intervention strategies (drugs or vaccines) that would protect people from this deadly disease.

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CHAPTER 4
The biology of malaria transmission

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Purpose

For the purposes of this text, *transmission* is defined as the progression of *Plasmodium* from a vertebrate host through the mosquito vector until inoculation into the next vertebrate host. Thus this chapter examines the biology of the infectious gametocyte in the vertebrate host, of the sexual and sporogonic development in the mosquito vectors, and of the delivery of infectious sporozoites into the proboscis of the mosquito.

History

Since the 1970s, the focus of malaria research has been on the cyclic development of the pathogenic asexual blood stages of *Plasmodium falciparum;* it is therefore perhaps necessary to recall that *Plasmodium* was first identified when Laveran saw the explosive emergence of the male gametes in a sample of blood taken from a soldier in Tunis (Laveran 1881). The appreciation of the role of these lashing cells was memorably summarized by Manson in his advice to Ross: "Follow the flagellum!" In so doing, Ross discovered that it was mosquitoes that are the most common vectors of *Plasmodium* (Ross 1897), and Grassi and colleagues recognized that *Anopheles* transmitted the malaria parasites to humans (Grassi 1900). McCallum soon recognized that the flagellum was indeed the male gamete and that fertilization took place in the mosquito, resulting in the formation of a motile "vermicule" (MacCallum 1897), thus making the vectors the definitive hosts of *Plasmodium*.

Recognition of the key role of the mosquito in transmission led to the early and very effective implementation of environmental, housing, and personal schemes to reduce human–vector contact and the early introduction of both residual and targeted household insecticide spraying campaigns, notably in the protection of colonial forces. The success of the introduction of DDT into such campaigns is difficult to overestimate.

The organization of national antimalarial campaigns, often by the military, resulted in the collation of large datasets that were very rapidly analysed and condensed into powerful equations to describe transmission. Not least among these were the studies of Ross himself, later developed by MacDonald into what is now described as the Ross–MacDonald formula (Smith 2012),

$$R_0 = \frac{ma^2 b}{r\left(-\ln p\right)} p^n \tag{1}$$

Advances in Malaria Research, First Edition. Edited by Deepak Gaur, Chetan E. Chitnis, and Virander S. Chauhan.

Where R_0 is the basic reproduction rate of the parasite under steady-state conditions, expressed as the number of secondary cases arising from each primary case; m is the mosquito-to-human ratio; a is the daily mosquito biting rate; b is the probability a sporozoite-infected mosquito will infect the vertebrate host; p is the daily probability of (infected) mosquito survival; n is the number of days taken for the mosquito to become infectious; and r reflects the probability a vertebrate host will infect the mosquito vector.

The growing understanding of how the biology of the parasites and their hosts affect each of the component variables in this formula may facilitate the future design and implementation of new and effective transmission-blocking interventions that can be applied in the diverse endemic situations encountered worldwide.

The current research agenda

The significant reductions in malaria distribution achieved during the Global Malaria Eradication Campaign of 1955 to 1969 in, for example, Australasia, the Mediterranean, and South America, have further improved in many countries. The reasons for the recent improvements are, however, diverse and sometimes unexplained (Cibulskis and Gosling 2012). The recent focus of malaria intervention has been to reduce both the acquisition of infection (notably through the use of insecticide-treated bednets) and the clinical impact of the disease by the administration of schizonticidal drugs. In the course of these endeavors, less attention has been paid to the impact of the newly developed interventions against the transmission of the parasite from the infected and treated host to the mosquito vector. Indeed, the knowledge that chloroquine (the one-time lead schizonticide) might temporarily enhance the infectivity of treated persons to mosquitoes (reviewed in Butcher 1997) might have changed previous treatment strategies.

Fundamental reevaluations of the malaria research agenda have been undertaken (Alonso 2011; Feacham 2009). Perhaps the single most important conclusion reached in many of these is that both interventions and research policy must take a more balanced approach, and recognize that the clinical prerogative, namely to alleviate disease in infected persons, should be complemented by efforts to reduce the number of secondary infections to a level below that which will sustain the parasites in a population (i.e., R_0 must be reduced to less than 1). For this to be achieved, we must bring together a much deeper understanding of the biology of the parasites and their interactions with both the vertebrate and insect hosts at all levels, from populations (actual and modeled) to the molecular.

This review attempts to identify what are currently believed to be key aspects in these sciences in the hope that this knowledge may promote the development of rational and sustainable transmission-blocking interventions. In preparing this analysis, it is recognized that as ongoing research fills past lacunae of knowledge, priorities and opportunities will change.

Biology

Parasite

Gametocytogenesis

We begin at the point when gametocytes are formed in the peripheral circulation of the vertebrate host. For the majority of malarial parasites, with the exception of *P. falciparum* and *Plasmodium reichenowi,* gametocyte induction and maturation are essentially concurrent with the growth and

replication of the asexual blood-stage parasites; by contrast, the peak gametocytemia in falciparum malaria occurs some 8 to 10 days after the peak asexual burden. In the intervening period, immature falciparum gametocytes are sequestered in the bone marrow (Smalley 1981; Thomson 1914; Tibúrcio 2012; Thomson and Robertson 1935), possibly resulting from nothing more than the physical rigidity of the immature cells (Aingaran 2012), caused by the development of an extensive subpellicular microtubular cytoskeleton (Sinden 1982). It has also been suggested that the physical retention of the immature gametocytes may be augmented in the stage I and II forms by sequestration to host CD36, possibly by the expression of the type C subset of PfEMP1, STEVOR, or RIFIN proteins on the giRBC surface (Sharp 2006; Bousema and Drakeley 2011).

The regulation of gametocyte differentiation has been described extensively at levels of light microscopy (Hawking 1971; Carter and Miller 1979), electron microscopy (Sinden 1978), and most recently at the molecular level (transcription and translation) (Sinden and Smalley 1979; Eksi 2012; Fivelman 2007; Silvestrini 2005; Young 2005). Studies have shown that expression of the transcription regulator *PfAP2-g* is essential for the formation of gametocytes (Llinas 2012). Prior to the first obvious morphological differentiation of gametocytes (stage II), a key role has been identified for *Pfgdv1* in the subsequent control of expression of eleven genes (Eksi 2012), seven of which are reportedly gametocyte specific: *Pfg27, Pfs16, Pfg14.744, Pfg14.748, Pfs47, Pfmdv1, and Pfgeko*. A very practical correlation of these detailed and elegant studies is that for the first 6 days of their development, the sexual cells are sensitive to most antimetabolites (Sinden and Smalley 1979), and to many schizonticides (e.g., chloroquine and artemisinin combination therapy (ACT) (Delves 2012a; Eksi 2012), but thereafter the maturing population (stage III to V) progressively enters cell cycle arrest and becomes insensitive to most schizonticides (Sinden and Smalley 1979; Butcher 1997; Delves 2012; Eksi 2012). Nevertheless, the mature gametocytes remain sensitive to interventions targeting energy production (e.g., ATP), such as primaquine (Graves 2012; Lelièvre 2012).

One potential evolutionary advantage of the prolonged development of falciparum gametocytes is that they are significantly protected from the damaging host responses to the asexual blood stages (see later). At the level of intervention management this means that in persons with high asexual parasitemia who attend clinic and are treated with schizonticidal drugs, sequestered gametocytes (stage I and II) will also be killed, but the drug-insensitive stages III and IV will subsequently emerge, when mature, into the peripheral circulation and can infect a new cohort of mosquitoes. It is this small surviving population of parasites (or their products) that should be targeted by new transmission-blocking interventions. Consistent with this view, White (2012) has advocated the addition of a single low dose of primaquine to artemisinin combination therapies to prevent transmission of falciparum malaria.

Mature gametocytes

Mature gametocytes are haploid sexually dimorphic, terminally differentiated cells. The female gametocyte is comparable to the vertebrate egg – fully resourced for rapid development. The haploid nucleus contains a highly chromatic and electron-dense nucleolus-like structure, and the nuclear envelope is surrounded by pigment produced from the digestion of hemoglobin by the trophic immature gametocyte. The cytoplasm is preprogrammed for rapid and extensive protein synthesis, containing a ribosomal population and endoplasmic reticulum that are particularly well developed. A single netlike mitochondrion and a smaller apicoplast are distributed throughout the cytoplasm (Divo 1985). P granules containing foci of translationally arrested messenger RNA are similarly distributed in the cytoplasm (Figure 4.1) (Thompson and Sinden 1994). The translation of more than 370 different proteins is arrested by a DOZI (development of zygote inhibited)-mediated mechanism (Mair 2006). Many of these proteins are subsequently translated in the early (hours 0–5) of development of the macrogamete and zygote in the mosquito midgut. Perhaps the most intriguing question to be asked is,

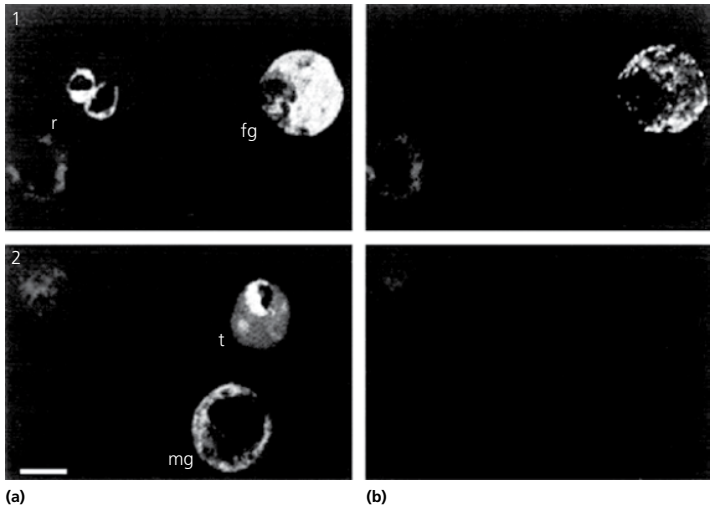

Figure 4.1 *In situ* hybridization to illustrate translational repression of a female specific messenger RNA. *1a* and *2a,* All parasites hybridize with a probe to the small subunit rRNA probe. r, ring stage asexual parasite; t, trophozoite; fg, female gametocyte; mg, male gametocyte. *1b,* Only the female gametocyte hybridizes with a Pbs28-specific probe, revealing a punctate cytoplasmic distribution of the stored messenger RNA. (From Thompson and Sinden 1994.)

"Other immunologically vulnerable proteins such as P48/45 and P230 are synthesized in the mature gametocyte. Why does the parasite maintain this complex mechanism to ensure that this set of 370 proteins, which include other transmission-blocking antigens such as P25 and P28, are *not* expressed in the vertebrate host?' One is tempted to suggest these proteins must be responsible for exquisitely sensitive functions of the parasite when it becomes extracellular in the blood meal inside the vector (see later).

The molecular/cellular makeup of the mature male gametocyte differs markedly from that of the female. The female proteome in *P. berghei* contains 19% (101/504) female-specific proteins, and the male contains 36% (236/650) male-specific proteins, including substantial quantities of (monomeric) α-tubulin II in the cytoplasm (Khan 2005; Hall 2005). The nucleus of the male cell, like that of the female, contains a haploid (1C) genome – though early cytochemical studies suggested it might be greater than 1C (Cornelissen 1988; Janse 1989). It may be that chromosomal protein structure in the terminally arrested gametocytes differs significantly from that of the asexual blood stages, thus distorting the comparative measurement of DNA content using histochemical methods. The nucleus of the male is significantly larger than the female, and it is multi-lobed; at the light-microscopic level this is seen as a more-dispersed distribution of the pigment. One nuclear pore is modified into a putative spindle pole body. On the nuclear face of the pore is an expanded electron-dense structure within which lie all the centromeres/kinetochores, and the telomeres of the otherwise invisible 14 interphase chromosomes lie distributed in the nuclear envelope. No other structures have been described in the nucleus. The cytoplasm of the mature microgametocyte has a very sparse ribosome population, virtually no endoplasmic reticulum, a small mitochondrion, and an apicoplast. In *Plasmodium*, unlike *Haemoproteus* (Sinden 2010), there is no evidence for a centriolar pinwheel structure on the cytoplasmic face of the nuclear pore on which the putative spindle plaque is found. At the time of writing (2012) there is as yet no evidence that the male has a similar abundant population of translationally repressed mRNAs (though this may be a quantitative rather than a qualitative statement).

The vast majority of gametocytes die within the host's blood stream, where they will be ingested by phagocytes and their constituent molecules presented to the host's immune system. Thus, in repeatedly infected individuals, the gametocyte-infected erythrocytes may be targeted by the immune effector mechanisms of the host. Notable among these targets might be the STEVOR and RIFIN protein classes that span the plasma membrane of the infected erythrocyte (Bousema 2011). In most *Plasmodium*, the gametocyte half lives are not significantly different (~110%) from that of the asexual blood stages, whereas in the genus *Laverania* the half life of mature gametocytes is variously estimated as 3.4 to 6.4 days (Smalley and Sinden 1977; Eichner 2001; Bousema and Drakeley 2011). Whereas the presence of anti-falciparum gametocyte antibodies correlates with a lower gametocytemia 4 weeks after antimalarial treatment (Saeed 2008), it would be fascinating to know whether the lifespan of the *g*ametocyte-*i*nfected *r*ed *b*lood *c*ell (giRBC) is reduced in hosts exhibiting significant responses to sexual stage antigens.

Often asked but unresolved questions are, "Does the blood taken by venipuncture truly represent the gametocyte population accessible to the vector in the dermal capillary bed?" and "Does the peak distribution of the gametocytes in the peripheral blood stream show a circadian, possibly crepuscular, pattern?" Although many observations on *Plasmodium* of all species suggest the mature (infectious) gametocytes are evenly distributed throughout the peripheral circulation, a considerable body of literature contests this assumption. Early studies suggested that *P. falciparum* gametocytes were more abundant in scarified skin samples compared to venipuncture (Chardome and Janssen 1952; van den Berghe 1952), and studies on *P. chabaudi* demonstrated higher gametocyte numbers in the mosquito blood meal compared to venous blood samples (Gautret 1996).

Data from *Leucocytozoon smithi* provide compelling evidence that the peripheral release of gametocytes can be tightly regulated by the day–night cycle acting through the pituitary gland of the turkey host (Gore and Noblet, 1978; Gore 1982). Hawking and colleagues (Hawking 1968; Hawking 1971; Hawking 1972) and Garnham and Powers (1974) together reported that gametocytes of *P. vinkei P. chabaudi, P. berghei, P. gallinaceum, P. coatneyi, P. knowlesi, P. cathemerium,* and *P. cynomolgi* all display short-lived maturity and infectivity to the mosquito. Although the combination of short-lived gametocytes and the tight synchronization of schizogony in the founding population of asexual parasites might explain the above data, the rarity with which such perfect synchrony is observed suggests other mechanisms may be at play. Among these is the severe impact of host factors (notably cytokines and leukocyte activation) generated in response to the release of parasite debris (e.g., pigment, and GPI anchors from parasite membrane proteins) at successive rounds of schizogony, upon the ability of microgametocytes to exflagellate (Motard 1990; Motard 1993; Targett 1994). A more challenging interpretation is required to explain the observation of Hawking and colleagues (1971), who demonstrated a diurnal periodicity in the ability of the long-lived *P. falciparum* gametocytes to exflagellate. Others have failed to demonstrate periodic infectivity (Bray 1976) or to consider the phenomenon unresolved (Gautret 2001).

Gametogenesis

When ingested by the blood-feeding vector (which for *Plasmodium* species could be an anopheline or culicine mosquito or a sandfly), the gametocytes rapidly detect multiple new environmental inducers (Figure 4.2). For the malaria parasites of mammals these are a temperature fall of more than 5 °C (Sinden and Croll 1975) and the presence of elevated concentrations of xanthurenic acid, an excretory product that in the mosquito results from synthesis of the ommochhrome eye pigment (Billker 1998). The latter can be replaced *in vitro* by raising the pH_{ext} to about 8.0 (Carter and Nijhout 1977; Kawamoto 1993). The role of temperature, if any, in the induction of gametogenesis of the malarial parasites of poikilothermic vertebrate hosts, such as lizards and snakes, remains to be resolved. These inducers interact with one or more receptors that stimulate, via a cGMP-dependent

Figure 4.2 Sequential development of *Plasmodium* in the mosquito vector. 1 and 2, Male and female gametocyte ingested with blood meal (0–30 min); 3 and 4, male and female gametes (15–60 min); 5, zygote (15 min–~9 h); 6, Ookinete burrows through the midgut epithelium (9–36 h); 7, Oocyst develops under the basal lamina of midgut, (days 1–25); 8, Rupture of oocyst, releasing sporozoites into the hemocele (days 9–21); 9, Salivary gland sporozoites (about day 10 until death of mosquito). Figure adapted from Billingsley and Sinden (Billingsley & Sinden, 1997).

protein kinase (McRobert 2008), a complex pathway of intracellular signals. Cytoplasmic Ca^{2+} controls the expression/de-repression of synthesis of P28 and other DOZI-repressed mRNA species in the female (McRobert 2008), and the rounding-up (disruption of the inner membrane complex skeleton, possibly by aspartic protease) of both the male and female gametocyte. In the male, CDPK4 activation is a prerequisite for DNA synthesis, axoneme polymerization, and mitosis (Billker 2004); thereafter, MAPK2 regulates chromosome condensation and initiation of motility by the microgamete flagellum, cytokinesis, and gamete release (Tewari 2005). Escape of both males and females from the host erythrocyte is mediated by proteases, resulting in the sequential dissolution of the parasitophorous vacuole (inhibited by E-64d) and the erythrocyte plasma membrane (inhibited by TLCK: N-alpha-tosyl-L-lysinyl-chloromethyl ketone) (Sologub 2011).

Gametogenesis can be completed within the amazingly short period of 15 to 30 minutes in most *Plasmodium* species. The resulting extracellular female gamete is spherical and nonmotile, and its surface coat, although inheriting pre-synthesized proteins, within 2 hours (Winger 1988) expresses proteins such as P25 and P28 newly translated from the stored mRNAs (Figure 4.2). The completed production of eight microgametes from each male gametocyte is revealed by the spectacular process of exflagellation (as originally observed by Laveran (Laveran 1881) (Figure 4.3). In the brief period following activation, the male gametocyte undergoes three sequential intranuclear mitoses. Simultaneously and in the cytoplasm one newly assembled centriolar pinwheel replicates three times to produce eight basal bodies upon each of which axonemes, 14 μm long, are assembled. Because the originating centriole in the gametocyte was physically connected through a nuclear pore with the intranuclear genome, there ensues a near balletic, coordinated three-fold duplication and redistribution of the resultant eight axonemes and the eight copies of the genome (Sinden 1976). Only at the culmination of this process do the axonemes become motile in the cytoplasm and swim out of the surface of the cell backwards, each basal body dragging a linked haploid genome, enveloped in a fragment of the original nucleus, into the gamete. The male gametes lack both

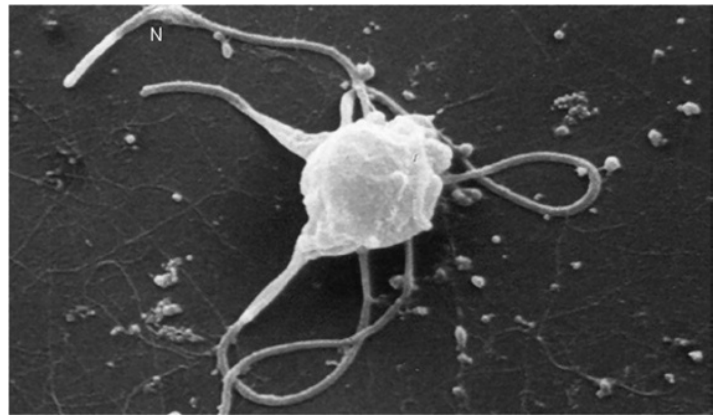

Figure 4.3 Scanning electron micrograph of *Plasmodium yoelii nigeriensis* microgametocyte undergoing the process of exflagellation, releasing the sometimes nucleated (N) male gametes. (Image from Sinden 1975a. Reproduced with permission of CNRS)

mitochondrion and apicoplast (Sinden 1976; Okamoto 2008), and inheritance of these organelles is therefore strictly maternal (Creasey 1993). The motility of these "sperm" is unsurprisingly driven by glycolysis (Wass 2012) and is short-lived (~40 min *in vitro*).

Fertilization is initiated by the recognition of the female by the male through the formation of a complex between P47 on the female and P48/45 on the male, both molecules interacting with P230. Interestingly, the sustained expression of P47 on the subsequent ookinete stage is believed to be the molecular target of mosquito complement (TEP)-mediated melanization of selected lines of *P. falciparum* in resistant mosquitoes (Molina-Cruz 2012). Gamete–gamete recognition is then followed by HAP2-mediated fusion of the plasma membranes of both cells and the total incorporation of the swimming male gamete into the female's cytoplasm (Liu 2008; Hirai 2008; Mori 2010). Following fertilization, as little as 0.1% of the macrogametes successfully mature into ookinetes, and of these about 1% form oocysts; the efficiency of each of these transformations is density dependent (Sinden 2008). Within about 12 hours, nuclear fusion has occurred and the diploid nucleus has, under the control of NEK2 and NEK4 (Reininger 2005; Reininger 2009), completed meiosis (Sinden 1991b; Sinden and Hartley 1985b) (Figure 4.4). The resulting single nucleus therefore contains either one haplotype (if resulting from self-fertilization) or maximally four haplotypes (if resulting from cross-fertilization). This, combined with the often low number of resulting oocysts, must be embraced in developing an understanding of the genetic complexity of parasite populations.

Following meiosis, the zygote transforms into a single motile ookinete in a process highly reminiscent of merozoite or sporozoite budding. Mediated by AP2-O (Yuda 2009), transcription of a new array of genes is initiated, it would be fascinating to know if this represents the first time that paternally inherited genes participate in development of the zygote – an event that in many eukaryotes can result in the uncovering of maternal/paternal genetic incompatibilities and the early death of the zygote. The mature ookinete possesses a uniquely complex set of rigid anterior organelles (Figure 4.5) (Garnham 1962; Garnham 1969; Canning and Sinden, 1973a; Davies 1974) that facilitate invasion of the mosquito midgut epithelium and associated physical barriers (the microvilli-associated network and peritrophic matrix (Zieler 2000)).

At the anterior tip, and through a single opening in the subpellicular skeleton (collar, apical, ring, and membrane vacuole), ducts from the numerous micronemes and other secretory vesicles can export a variety of molecules that are responsible for providing purchase of the parasite on its many

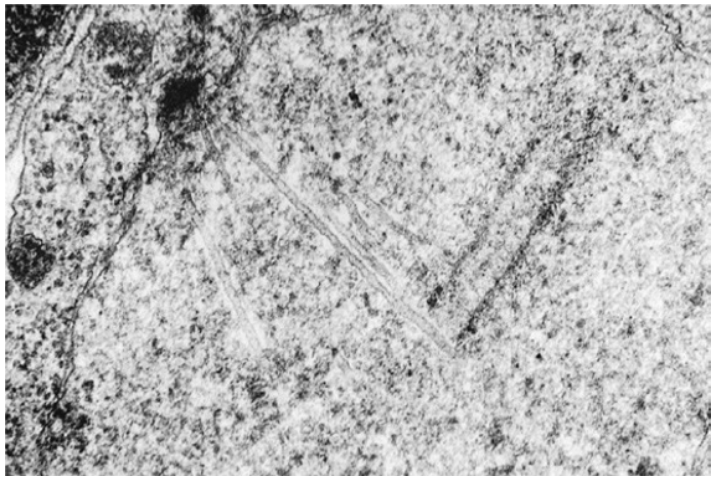

Figure 4.4 Transmission electron micrograph of *Plasmodium yoelii nigeriensis* zygote illustrating a synaptonemal complex diagnostic of the leptotene and diplotene stages of meiosis. (Image from Sinden and Hartley 1985.)

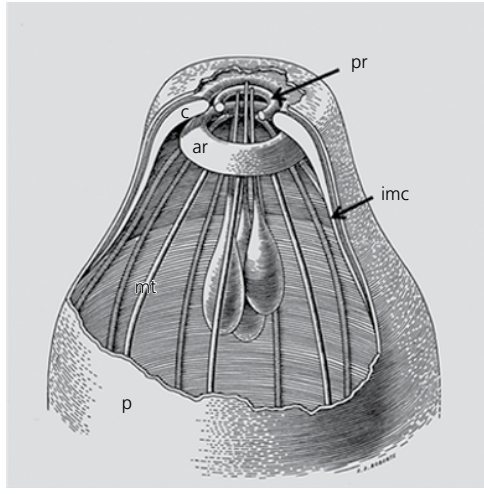

Figure 4.5 Reconstruction of the apical complex of the *Plasmodium berghei* ookinete. p, plasma membrane; c, collar; ar, apical ring (the organizing center for the microtubular cytoskeleton); mt, subpellicular microtubules, pr, polar ring; imc, inner membrane complex (see Figure 4.6). (Image from Sinden 1973a.)

substrata (e.g., CTRP [circumsporozoite thrombospondin-related protein]), for defending the parasite against protease attack (e.g., P25, P28), crossing the peritrophic matrix (e.g., chitinase), for recognizing midgut moieties (e.g., enolase-plasminogen) (Ghosh 2011), for recognising the midgut epithelial cell (unknown), for disrupting the midgut epithelial cell (e.g., POSH, SOAP, PSOP (Ecker 2008; Dessens 2003)), and mediating migration across the epithelium (CelTOS (Kariu 2006)).

The molecules on the epithelial cell that are recognized by the ookinete are as yet incompletely understood. Early studies identified essential carbohydrate moieties (Zieler 1999), and evidence has been found that many (five) ookinete-interacting molecules are found in the detergent-rich moieties (lipid rafts) from the epithelium, including APN1 and a secreted glycoprotein of unknown function (AgSGU) (Jardin, unpublished data). The ookinete, like other motile stages, possesses a

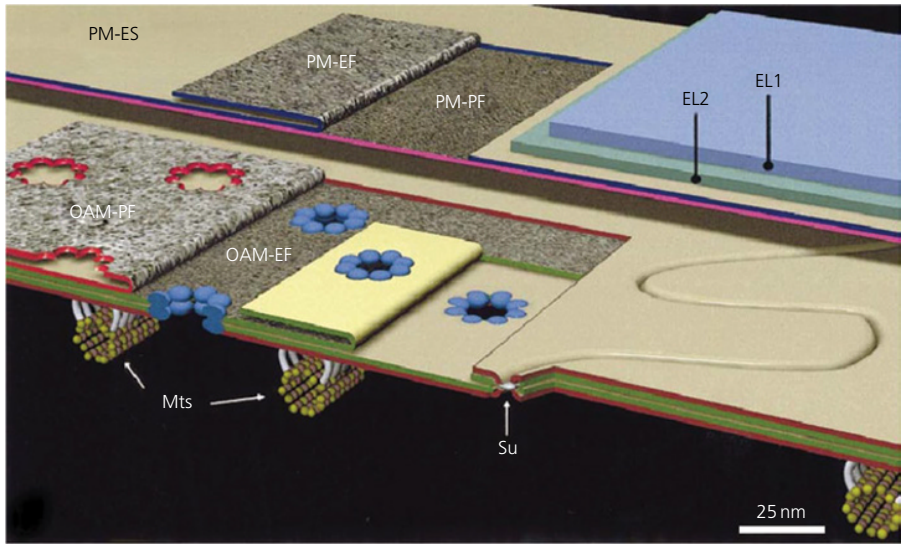

Figure 4.6 3D reconstruction of ookinete pellicle based upon cryofracture images of *Plasmodium gallinaceum*. EL1 and 2 external layers on the cell surface; PM-ES, plasma membrane external surface; PM-EF, plasma membrane external face; PM-PF, plasmalemma protoplasmic face; OAM-PF, outer alveolar membrane protoplasmic face; OAM-EF outer alveolar membrane external face; mts, microtubules; su, suture between adjacent edges of the alveolar vacuole membrane. Note the 8-fold symmetry of pores in the inner membrane complex. (Image from Raibaud *et al.*, 2001.)

glideosome motor (Wass 2012) within the pellicle (Figure 4.6) that, under the control of cGMP and Ca^{2+} drives the ookinete at about 4 μm/min along its corkscrew path through the hostile blood meal toward the midgut wall (Moon 2009; Siden-Kiamos 2006). Invasion of the midgut epithelial cell invariably results in the lysis of the latter and its expulsion from the epithelium (Zieler and Dvorak, 2000; Han and Barillas-Mury, 2002) (Figure 4.7).

What molecular/physical cues the ookinete recognizes as it emerges from the disrupted epithelial cell and contacts the collagen-rich basement membrane remain unknown, but it is here that the ookinete invariably ceases moving and differentiates into a vegetative oocyst. It is during the traversal of the epithelium that in refractory mosquitoes, many ookinetes are either lysed or melanized (Povelones 2011) (Figure 4.7). Genetically attenuated (noninvasive) ookinetes inoculated directly into the hemocele can mature in the hemocele (Ecker 2007), where they may be found attached to the basal lamina of many organs and tissues including not only the gut but also the Malpighian tubules, strongly suggesting a molecular recognition and attachment to these laminae. The majority of ookinetes that successfully escape the blood meal do so within 24 to 36 hours after blood meal ingestion, delayed parasites are digested by the midgut trypsin-like enzymes (Gass, 1977; Gass and Yeates, 1979).

Oocyst formation

Oocysts normally grow and differentiate under the basal lamina for a temperature- and species-dependent period of 9 to 15 days, increasing in size (from 5 to ~50 μm diameter), biomass, and genome copy number (from 4N to 500–4000N) (Figures 4.8 and 4.9). The process of oocyst–sporozoite development (sporogony) at the morphological level closely resembles schizogony in both the liver and blood-stage parasites (Sinden 1978), indeed so much so that I was told by P.C.C. Garnham that the first time he saw the pre-erythrocytic schizont in liver sections he was positively reassured by its similarity to an oocyst! We still have a very poor knowledge of molecular events involved.

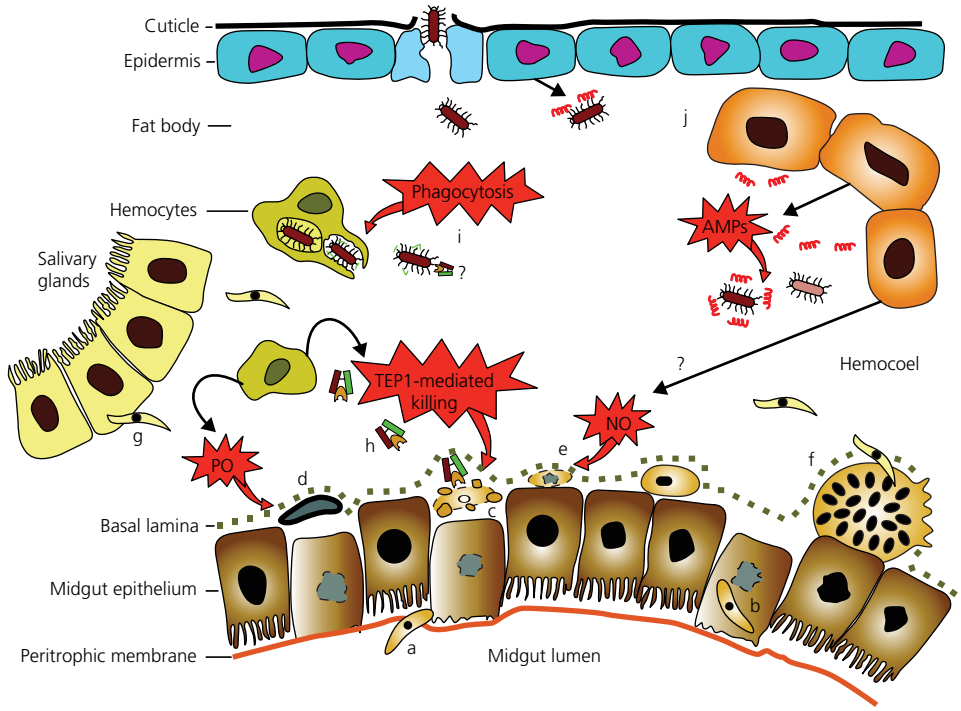

Figure 4.7 Summary of known immune mechanisms used by the mosquito to attack malaria parasites. Ookinetes (a) can be killed by expulsion of the invaded midgut cell, by LRIM/APL1C/TEP-mediated lysis (c) and phenol oxidase-mediated melanization (d) in the basal labyrinth. Oocysts can be killed by NO (e). Melanization of oocysts to form Ross's black spores is not illustrated. (Image from Yassine and Osta, 2010.)

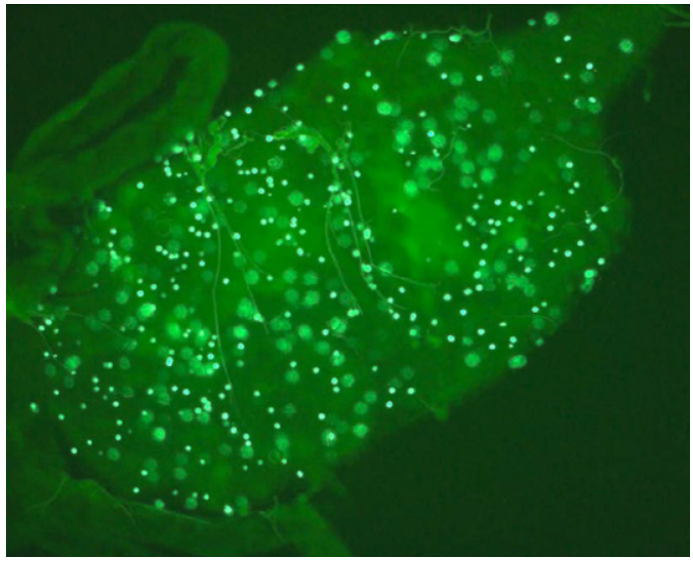

Figure 4.8 Image of dissected midgut of *Anopheles stephensi* infected 5 days previously with a GFP-expressing clone of *Plasmodium berghei*. Note that oocysts on one side of the gut appear small (in focus) and on the other side large (out of focus). Oocyst numbers in such images are readily counted by simple algorithms. (Image from Delves and Sinden, 2010.) (*See insert for color representation of this figure*.)

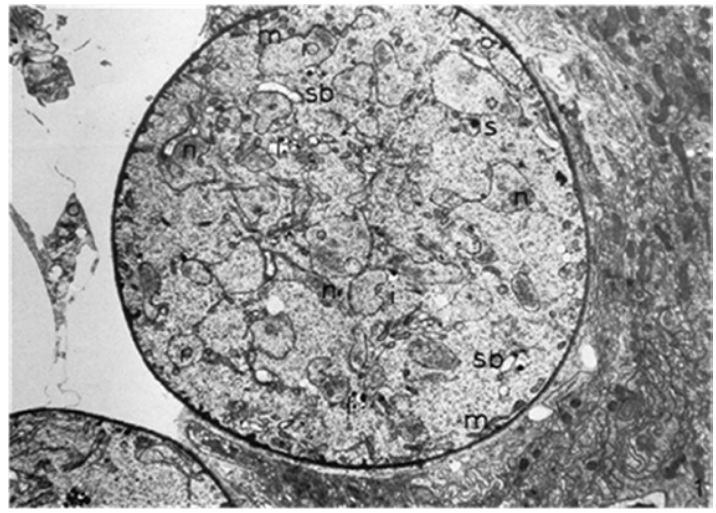

Figure 4.9 Transmission electron micrograph of 6- to 7-day *Plasmodium falciparum* oocyst in *Anopheles gambiae*. Nuclear profiles contain 'nucleoli' (n) and synchronous spindles (s) originating in characteristic invaginations of the nuclear envelope (i). Mitochondrial (m) and apicoplast (here termed spherical bodies, sb) profiles are seen in the cytoplasm. The electron-dense cyst wall is distinct from the paler-staining basal lamina of the midgut wall. (Image from Sinden and Strong, 1978.)

Molecules expressed during oocyst development identified to date include Cap380, CSP, ECP1, IMC1a, LAPs1-6, and transglutaminase (reviewed in Aly 2009), and transcription of 47 genes (termed *uos: u*pregulated in *o*ocyst *s*porozoites) is significantly upregulated (Mikolajczak 2008). Ongoing transcriptomic and proteomic studies may be expected to expand this repertoire and to reflect not only the broad ultrastructural conservation of vegetative growth but also rare and specific modifications that have evolved in the adaptation to hazards and opportunities in the insect host. Among the first of these adaptations to be identified at the ultrastructural level is the development of cristae in the mitochondrion. Atovaquone, an inhibitor of the cytochrome bc_1 complex (ubiquinol: cytochrome *c* oxidoreductase, respiratory Complex III), a key enzyme of the mitochondrial electron-transfer chain, is exquisitely active against the ookinete stages in the mosquito midgut (Fowler 1995). Phenotypic analysis of gene knockouts has demonstrated that the expression of many gene products (e.g., the LAP/CCCP protein family) can be detected in the zygote and ookinete stages, yet the developmental consequences are only revealed at the morphological level, when numerous developmental pathways converge upon sporozoite formation (Raine 2007; Ecker 2007; Sinden and Matuschewski, 2005).

Immediately prior to sporozoite formation (Figures 4.10 to 4.12) the numerous peripherally distributed haploid daughter nuclei (or possibly lobes of a large syncitial nucleus) undergo the final mitotic division. Each of the daughter spindle poles organizes, in the cytoplasm of each sporozoite bud, the assembly of the anterior secretory micronemes and rhoptries, of the inner membrane complex, of the glideosome motor, and of the apical microtubule organising center (MTOC), which in turn organizes the assembly and patterning of the polarized longitudinal microtubule cytoskeleton (Sinden 1978; Schrevel 2008). The progressive assembly of the IMC and microtubules apparently occurs at the annular nucleating center located at the junction of the bud and the body of the sporoblast. The progressive assembly of the inner membrane complex and microtubule cytoskeleton determines the elongate shape of the sporozoites as they bud from the surface of the sporoblast (Figures 4.10–4.12).

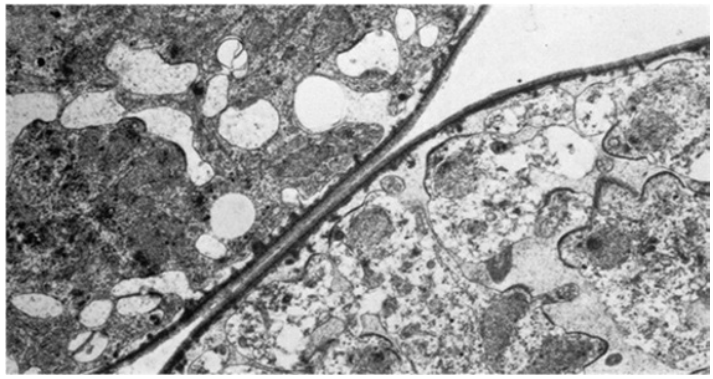

Figure 4.10 Transmission electron micrograph of 10-day *Plasmodium falciparum* oocysts in *Anopheles gambiae*. Sporoblast formation begins (top left oocyst) by expansion of the lumen of the endoplasmic reticulum; thereafter the mitotic nuclei are located immediately beneath the sporoblast plasma membrane, and the sporozoites form by outgrowth from the cell surface. (Image from Sinden and Strong, 1978.)

Although they are immotile during their assembly (Figure 4.13), following cytokinesis the sporozoites become motile within the oocyst. This motility, combined with the secretion of cysteine proteases (e.g., ECP1) and CSP (Wang 2005) and the constant churning of the midgut musculature, results in the progressive thinning and eventual perforation and/or catastrophic rupture of the enveloping cyst wall (Sinden 1974; Aly and Matuschewski 2005) (Figure 4.14). The previously impenetrable basal lamina has by this time become stretched by the expanding oocysts into a lace-like network of filaments, which offers no barrier to the small (12 μm × 1 μm) emerging sporozoites (Sinden 1975b). Whereas a minority of released sporozoites can be found in the cytoplasm of midgut epithelial cells or the midgut lumen, the vast majority of liberated sporozoites are carried in the hemocelomic fluid through the ostia in each abdominal segment into the heart, from where they are pumped anteriorly through the aorta into the thorax (Hillyer 2007a), where they may meet the basal lamina of the salivary glands.

The surface protein CSP binds via its central region I to the basal lamina (Sidjanski 1997), TRAP-A domain interacts with sagalin (Wengelnik 1999; Ghosh and Jacobs-Lorena 2009), and UOS3, TREP, and MAEBL bind with unknown ligands (Mikolajczak 2008). The proteins PCRMP1 and PCRMP2 play essential but unknown roles in salivary gland invasion (Ghosh and Jacobs-Lorena 2009). The now-attached parasites invade the subtending salivary glands (Pimenta 1994). The specificity of this initial interaction results in the sporozoites preferentially accumulating in the central and distal lateral lobes of the glands. Here they form, and enter, a transient vacuole from which they escape and invade the cytoplasm of the cells, progress through the acinus, and move into the salivary duct (which it is not chitinized in the selected lobes of the glands (Janzen and Wright 1971; Pimenta 1994) (Figure 4.15).

Invasion of the salivary glands provokes upregulation of immune response proteins, notably defensin, GNBP, and IGALE20 (Dimopoulos 1998) and serpin 6 (An 2012). The sojourn of the sporozoites in the gland can be very long. Boyd (1949) describes infectious residencies of 40 and 56 days, even stating "Some specimens remained alive and infective for six months." Within the glands the sporozoites differentiate further. They lose the ability to reinfect the salivary glands (Vanderberg 1975; Vanderberg 1974) and become more infectious to the vertebrate host (Touray 1992) – a developmental transition that is accompanied by the upregulation of transcription of 141 new proteins (Mikolajczak 2008), including UIS 3 and 4 (Matuschewski 2002), which may be post-translationally regulated by SAP1 (Aly 2009) and have important roles in subsequent hepatocyte infection.

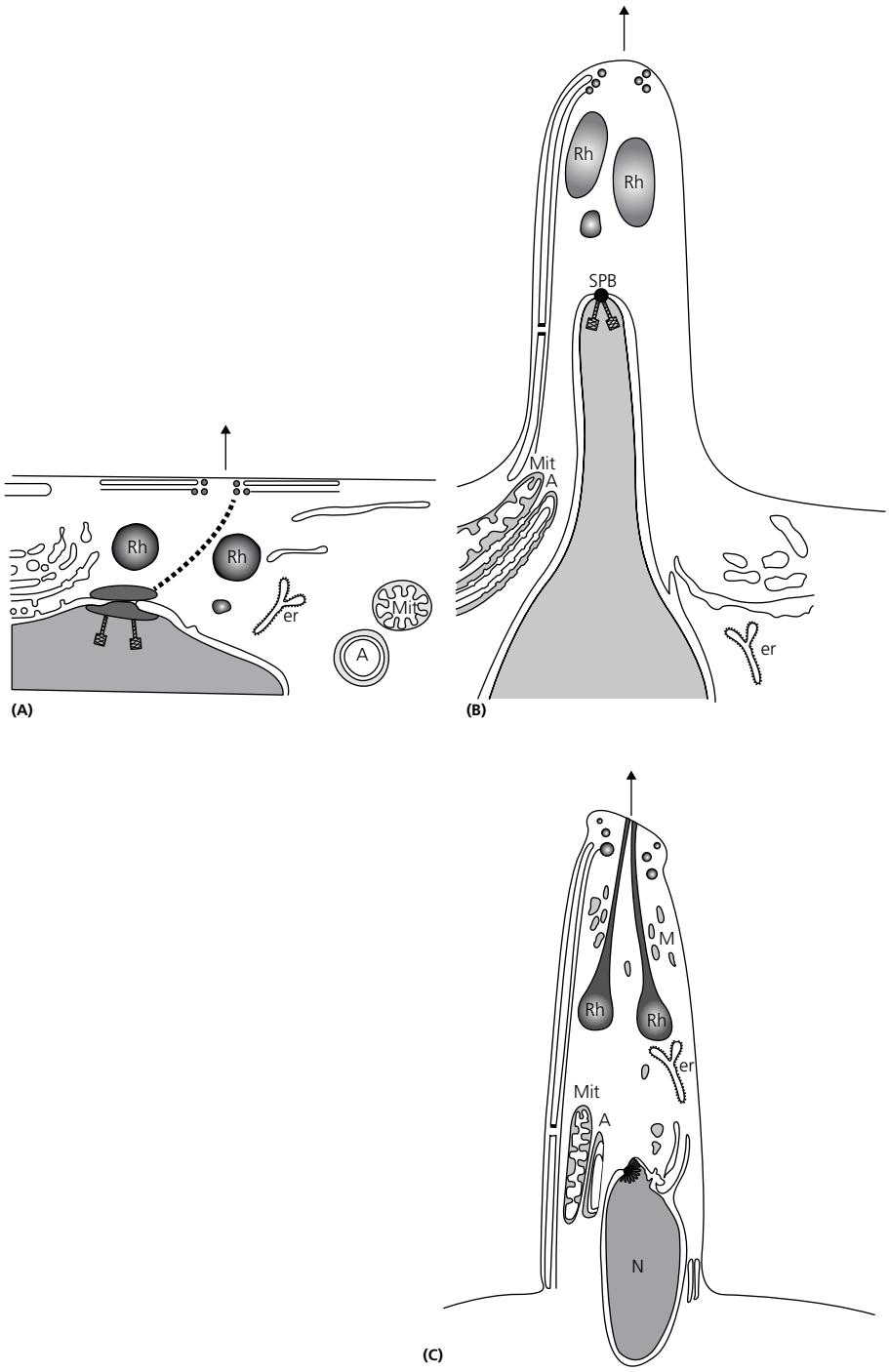

Figure 4.11 Sequential steps in sporozoite formation in the oocyst. *A*, Vacuole of inner membrane, and vesicle precursors of rhoptries (Rh) and micronemes laid down under plasma membrane adjacent (linked?) to a pole of final nuclear mitotic division. *B*, Assembly and elongation of the IMC and microtubular cytoskeleton draws spindle pole into sporozoite bud. Mitochondrion (Mit) and apicoplast (A) move together into bud. *C*, Maturation of secretory vesicles, differentiation between rhoptry and micronemes detectable. (Image from Sinden and Matuschewski 2005.)

Figure 4.12 3D reconstruction of anterior pole from cryoelectron tomographs of sporozoite (cf. ookinetes, Fig. 4.3a). Purple, plasma membrane; green, microtubules; yellow, inner membrane complex; pink, rhoptry; blue, micronemes; brown, polar rings (MTOC). (Image from Kudryashev *et al.*, 2012.) (*See insert for color representation of this figure*.)

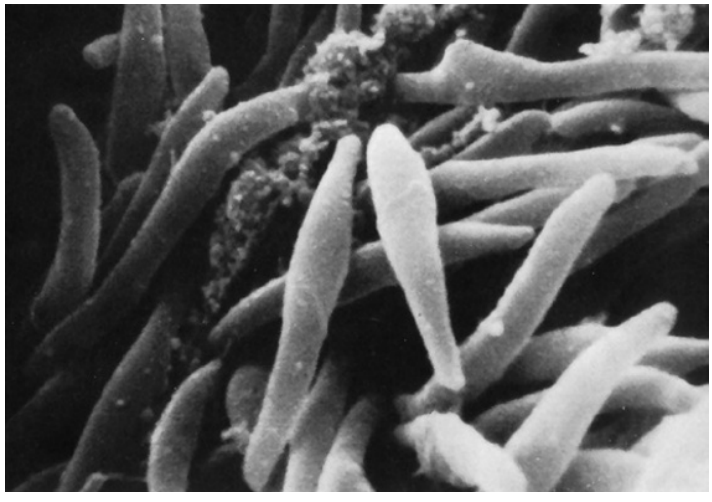

Figure 4.13 Scanning electron micrograph of *Plasmodium yoelii nigeriensis* sporozoites immediately prior to cytokinesis from the sporoblast body of the oocyst. (Image from Sinden, 1974. Reproduced with permission of CNRS.)

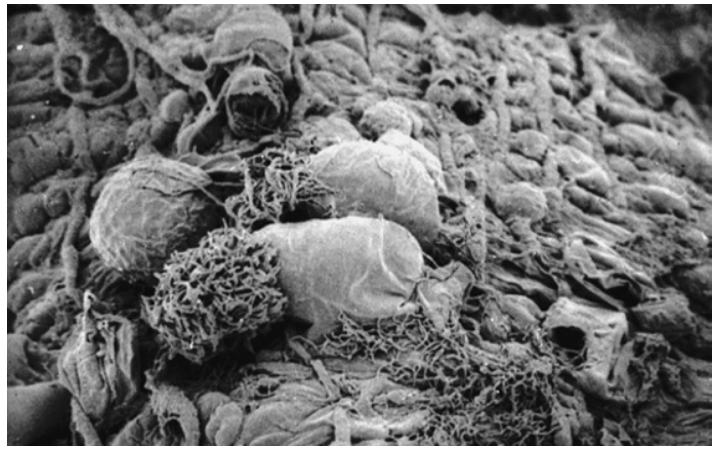

Figure 4.14 Scanning electron micrograph of *Plasmodium yoelii nigeriensis* sporozoites emerging from catastrophically ruptured oocysts on the midgut of *Anopheles stephensi*. (Image from Sinden, 1974b.)

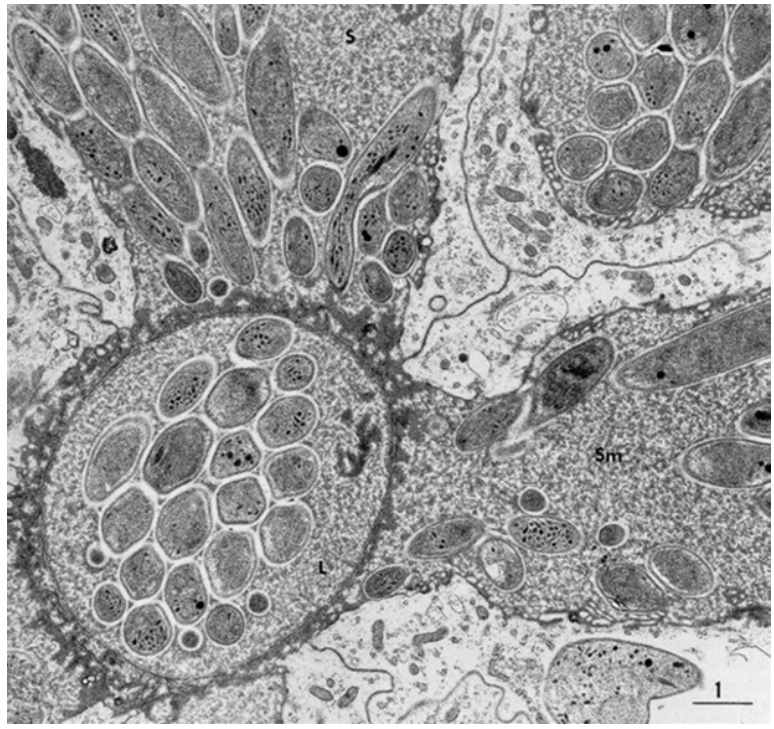

Figure 4.15 Transmission electron micrograph of *Plasmodium yoelii nigeriensis* sporozoites in the salivary glands of *Anopheles stephensi*. Note sporozoites lying free in the cytoplasm and the acinus of the cells (s, sm) and tightly packed in the, here partially chitinised, lumen (l) of the duct of the gland from where they will be ejected into the skin of the vertebrate host. Picture taken from (Aikawa and Sterling, 1974).

Of the sporozoites released into the hemocele it is frequently estimated that 10% to 19% reach the salivary glands (Rosenberg and Rungsiwongse, 1991; Korochkina 2006). The attrition is rapid and is not explained by phagocytosis by hemocytes (Hillyer 2007b). This figure is, however, density dependent. In the rodent-model *P. berghei,* the salivary gland sporozoite population begins to saturate at about 10,000 per full gland set (Sinden 2008); in other parasite–vector combinations, 60,000 are commonly recorded and 219,450 described (Boyd, 1949). Salivary gland sporozoite numbers are enhanced if the mosquito takes a second bloodfeed following infection, and numbers peak 20 to 25 days after infection; thereafter, they fall progressively (Boyd 1949). Sugar-feeding and blood-feeding mosquitoes eject sporozoites (Billingsley 1991), thus contributing to these losses. It remains unclear whether sporozoite viability *per se* falls over time. Perhaps the biggest, and in many ways the most important, lacuna in our knowledge of malaria transmission is our lack of understanding of the relationship between sporozoite burden in the salivary gland and infection of the vertebrate host (see Population Dynamics, later).

Vector

Authoritative sources suggest that 41 of 70 anopheline species are major vectors of the human malarias worldwide (Sinka 2012). Although the consequent diversity of this number alone is challenging, it pales to insignificance when compared to the total number of mosquito species (147) and genera (9) considered to be vectors of *Plasmodium* species. In considering all malaria parasites, it must be recalled that vectors of avian malarias include culicine mosquitoes, and many of the ancestral saurian parasites (Ayala 1973; Schall 1990; Kimsey 1992) are transmitted by the equally primitive sandflies (and possibly mites) (Schall and Smith, 2006). The molecular foundations of these interspecies compatibilities/ and incompatibilities remain largely unexplored. The following comments are confined to anopheline vectors.

Malaria is transmitted by female mosquitoes because they alone take blood feeds, perhaps as frequently as every 3 to 4 days (Beier 1996). Although ookinetes fed to *Drosophila* do not infect the insect, if directly inoculated into the hemocele of this species, ookinetes (of *P. gallinaceum*) will mature into oocysts and release sporozoites that, when extracted mechanically, are infectious to the chicken host (Schneider and Shahabuddin 2000). This study failed to report whether sporozoites had infected the salivary glands of the insects. Thus, whereas sex-dependence of blood feeding and infection can be laid clearly at the door of exposure, we are left to ask why some mosquito species are more susceptible to infection by individual malaria species than others. Reasons could be intrinsic (genotype) or extrinsic (the complex microbial ecosystem of the female mosquito midgut; environmental factors), but in many experiments the recognition of and distinction between these possibilities have not been made clear.

Intrinsic factors

There is an extensive literature on the highly variable blood-feeding behavior of mosquito species relating to place (exophilly or endophilly), time (crepuscular, day or night time), blood source (anthropophilly or zoophilly) and biting-site preferences. Chemical and behavioral analyses of the attraction of the human host to the mosquito continue apace, but simply stated, long-distance attraction is achieved by mosquitoes flying upwind into the plume of CO_2 exhaled by the target organism. As the insect approaches the host, heat, moisture, and odor detection supplement the drivers directing the insect toward the potential blood source (Costantini 1996). Detailed chromatographic experiments have demonstrated that ammonia, lactic acid, and tetradecanoic acid and other volatiles, such as dimethyl disulpfide generated by bacteria (e.g., *Cornybacterium* (Verhulst 2011)) on the skin are among the key chemoattractants for *A. gambiae* – a mosquito

with pronounced anthropophilic feeding behavior. Genetic analysis has identified the roles of distinct classes of odorant receptors (AgOR) and ionotrophic receptors (AgIR) (Liu 2010) in vector behavior.

The whole question as to whether and how malaria parasites might manipulate their mosquito vectors has received rare and diverse attention. One review (Cator 2012) has drawn together much of the published literature, and it cautiously concludes that "the nature and extent of the phenomenon remains unclear," but mathematical modeling studies illustrated how behavioral modifications might induce changes in transmission levels as large as the impact of insecticide-treated bednets (ITNs). Evidence suggests malaria infection increases the probability of mosquito blood feeding (Day and Edman 1983a; Koella 1998; Anderson 1999; Rossignol and Shieh 1993) and that the presence of sporozoites in the salivary glands reduces the probability of surviving the blood-feed by as much as 37.5% (Anderson 2000). Yet other observations that sporozoite infection both increased the biting rate and probing time (Rossignol 1984b; Rossignol 1986b) might suggest that the infected mosquito causes greater irritation, therefore provoking a host reaction. The evolutionary advantage of this strategy to the parasite is alleged to be that the enhanced biting and probing increases the probability of parasite transmission but at the expense of vector survival. This and the fact that heavily infected mosquitoes have a reduced lifespan (Dawes 2009) suggest the impact of the small or the lightly infected mosquitoes on parasite transmission through populations may have been underestimated.

Immune responses

In light of the dramatic advances in our understanding of the intrinsic molecular relationships between the malarial parasites and their mosquito vectors, most notably of the immune responses involved, it is humbling to think now of the heroic classic descriptions of vector competence (Huff 1930; Weathersby 1985), observations that remained undeveloped for much of the last half century.

With the intervention of experts in *Drosophila* genetics and the innate immune system, combined with the unravelling of the *Anopheles* and *Aedes* genomes, things have moved forward apace. A framework of understanding now exists of the key roles of the innate immune system of the mosquito, its induction, and the mechanisms by which it modulates the biology of malarial infections (Dong 2011; Lowenberger 1999; Dimopoulos 1997; Osta 2004) (Figure 4.7).

The variability in vectorial capacity of natural populations of mosquitoes is unquestionably a key facet in understanding the global distribution of malarial parasites. With the benefit of hindsight, it is clear that the immune status of the insect is determined not only by the genotype of the mosquito but also by numerous geographic and environmental covariant external factors. It is necessary to understand the relative contributions of these in any individual analysis.

The complexity of potential genomic changes that could underlie variations in vectorial competence of *Anopheles* speies, such as G3 versus L3 to L5 lines (Collins 1986), far outweigh the fascinating observation that variation or knockdown of single genes such as *tep1* can determine in very large part the vectorial potential of species such as *Anopheles quadriannulatus* (for *P. berghei*) (Habtewold 2008). Elegant gene-silencing studies have shown that at least three immune pathways can suppress malaria infection in the mosquito (Figure 4.7) and that different infecting parasite species reportedly induce different immune responses in mosquitoes of the same laboratory population (i.e., with identical genotype and potentially identical extraneous factors). The cost that the insects pay in mounting these responses in terms of loss of fitness, survival, and fecundity are evidentially affordable but might not be insignificant (Chang 2012). It remains a matter of debate whether all mosquito species should be considered to be naturally or innately susceptible or resistant to malarial infection (Cirimotich 2011; Sinden 2004).

Extrinsic physical factors

Physical factors known to influence the success of malaria transmission through the mosquito include ambient temperature, humidity, and light regimens. Of these, the day/night/crepuscular patterns of mosquito blood feeding dominated early studies, not least because of their impact upon the management of insecticide campaigns. Night-feeding species include *Anopheles funestus* and *A. gambiae*; crepuscular feeding species include *Anopheles freeborni*.

Laboratory studies have permitted controlled studies on the influence of both mean temperature (Noden 1995; Vanderberg and Yoeli 1966; Sato 1996) and its diurnal fluctuation (Paaijmans 2010) on parasite transmission. Observations from the field clearly indicated that *P. falciparum,* for example, although readily transmitted in lowland (warm) areas, was not transmitted in adjacent highland (cool) areas (Boyd 1949). Unsurprisingly early laboratory studies then demonstrated the permissive temperature range for mosquito transmission of *P. falciparum* was 18 °C to 27 °C (<16 °C, >30 °C are nonpermissive) (Noden 1995; Boyd 1949). Conversely, sporogony of the rodent malaria parasite *P. berghei,* which is endemic in the highlands of Katanga, was optimal in the laboratory at the lower temperature range of 16 °C to 24 °C (Vanderberg and Yoeli 1966). Sato and colleagues (1996) further dissected this temperature profile and showed that it was gametocyte-to-gamete development (i.e., the first 30 minutes of sporogonic development) that was the most susceptible to temperature variation (21 °C viable; 26 °C lethal), as compared to subsequent oocyst-to-sporozoite progression (1 hour to 12 days), which tolerated temperatures of 26 °C to 27 °C. The general conclusion to be drawn is that temperatures below the optimum resulted in slowed development but did not kill the parasite, whereas temperatures greater than the optimum were lethal (Noden 1995; Sato 1996). It is tempting to ask whether infected mosquitoes in the field seek refuge from high ambient temperatures.

Extrinsic biological factors

Blood meal size has a major influence on the success of transmission. Clearly the larger the blood meal, the more gametocytes are ingested, and consequently the higher the probability of transmission. An additional benefit of a large blood meal is that it takes longer to digest (Briegel and Lea 1975; Feldmann 1990). It has been demonstrated that the malarial ookinete is sensitive to mosquito protease (Gass 1977; Gass and Yeates 1979), enzymes that show peak expression 24 hours after feeding (Muller 1993). Thus if the ookinete does not escape the blood meal quickly, it may be killed. It is therefore unsurprising that laboratory studies show that within a population of feeding mosquitoes, those less than 50% fully engorged are poorly infected, if at all, compared to their fully fed peers, where digestion rates will be slower.

Early field studies suggested that children of 3 to 14 years old, who can have the highest gametocyte densities in their peripheral blood stream, represent the major fraction of the infectious reservoir for *P. falciparum* (Hino 2012; Muirhead-Thomson and Mercier 1952a; Muirhead-Thomson and Mercier 1952b); nevertheless, there is now recognition that adults with low gametocytemias might represent a not-insignificant fraction (28%–38%) of the infectious reservoir. Persons with more than 101 gametocytes/μL reportedly infect about 25% of blood-fed mosquitoes, and even those with undetectable gametocyte numbers infected about 5% of mosquitoes (Bousema and Drakeley 2011; Bousema 2012).

An important area of interest is the impact of the diverse biota in the complex ecosystem of the blood-fed mosquito; that is, the impact of other species – symbionts, commensals, or parasites (viruses, bacteria, protozoa, microsporidia, algae, fungi, nematodes, parasitoids) – in the blood meal or tissues of the female mosquito (Cirimotich 2011). There is clear evidence that coinfecting organisms can affect transmission in any of four ways. First, they may compete for the finite nutrients available; second, they may reduce the longevity and fecundity of the blood fed mosquito;

third, they may modulate, through PGRP-LC signalling (Meister 2009), the innate immune responses of the mosquito responses that are known to cross-react with *Plasmodium* (Dong 2011); fourth, they may themselves produce products, such as reactive oxygen species (Cirimotich 2011), that kill *Plasmodium* directly. Such impacts might be more important with respect to bacteria such as *Enterobacter, Pseudomonas,* and *Serratia* and algae found in the blood meal, whose populations rapidly expand following ingestion of the blood meal (Boissière 2012) and may either inhibit the sensitive early stages of sporogony or cause the early death of the vector (~4 days after the blood feed (Dawes 2009)).

The variation in the composition of the blood meal ecosystem both in the field (Pumpuni 1996) and in the laboratory (Pumpuni 1993) is an often unrecognized confounder of the ability to directly compare the transmission of *Plasmodium* to mosquitoes in different localities and laboratories (Straif 1998). Whereas studies on vector susceptibility have focused primarily on the impact of bacteria in the blood meal (Dimopoulos 1997), studies on the important property of longevity of the infected mosquito have focused primarily on the impact of fungi such as *Beauveria* and *Metarhizium* (Blanford 2011). These studies exploited the attractive evolutionary concept that the late lethality of the fungal infection, while impairing onward parasite transmission, had little impact on mosquito fecundity (early egg production) (Thomas and Read 2007; Blanford 2011), thus exerting a lower selection pressure on the mosquito genome.

The impact of the highly entomopathogenic microsporidan parasites such as *Nosema* upon malaria transmission is significant, notably due to the attrition of the mosquito population. Interestingly, coinfections often revealed, within one mosquito, marked melanization of *Nosema* but not of the coexisting malaria oocysts or sporozoites (Hulls 1971; Canning and Sinden 1973b; Bargielowski and Koella 2009). Much less well studied are midgut algae, whose expanded populations in the infectious blood meal often correlate with a complete suppression of *Plasmodium* infections and simultaneous disruption of the mosquito gut epithelium (RE Sinden, unpublished observations).

Vertebrate host

Numerous physiological and behavioral parameters (locality; movement; odor; body size; heat; CO_2; biting habit) limit the potential interactions between the host and vector, but these lie outside the remit of this chapter.

It has been suggested that the rodent parasites *P. berghei, P. chabaudi,* and *P. yoelii* render the mouse host more susceptible to mosquito feeding by enhancing the probability that capillaries can be located by the mosquito (Rossignol 1985), and this at a time when gametocytes are most infectious. Additionally the parasite may render the host less responsive to the irritation of the bite (Day and Edman 1983a; Day and Edman 1983b; Rossignol and Shieh 1993). Recognizing that in these species, gametocyte abundance (and *potential* infectivity to the mosquito) parallel that of the asexual parasites, it might be assumed the more abundant asexual blood stages mediate this phenomenon. It would be interesting to know if the same phenomenon occurs in *Laverania* species where asexual and sexual parasite abundances are temporally distinct. It is difficult to reconcile the above concepts with the study of Dearsly and colleagues (1990), who report that infectivity of *P. berghei* gametocytes is highest at day 3 after blood infection, whereas both gametocyte and asexual parasite numbers peak between days 10 and 15. This work is consistent with studies on *P yoelii* and *P. vivax,* suggesting that the host response to schizogony suppresses gametocyte infectivity (Motard 1990; Motard 1993; Targett 1994; Naotunne 1993; Sinden 1991a).

It is beyond doubt that the adaptive immune system of the naturally infected host can have a profound impact upon transmission of *Plasmodium* to the mosquito (Graves 1988; Bousema 2010; Roeffen 1989). Naturally induced transmission-blocking immunity occurs because the mature gametocytes in circulation in the peripheral blood stream of the host synthesize proteins that are

expressed on the surface of the gametocyte-infected erythrocyte (e.g., SURFINS and RIFINS), or later, on male or female gametes and zygote following their escape from the sanctuary of the host erythrocyte when in the blood meal of the vector (e.g., P230, P48/45), where, bathed in the plasma of the host, the antibodies ingested in the infectious blood meal can bind to the gamete's or zygote's surface. The location of these immunogens in or on the gametocyte-infected red cell is unlikely to be critical to immunogenicity because every gametocyte protein will be presented to the host's immune system. Greater than 99.99% of the gametocyte population die in the vertebrate host and are presented to the immune system through the spleen. It might be anticipated therefore that as a defense, proteins of this type that significantly affect transmission, such as P48/45 and P230, may be more polymorphic or variant than zgote and ookinete surface proteins that are translated *de novo*, following induction of gametogenesis, in the blood meal, such as P25 and P28. Although the relative conservation of the latter has been clearly demonstrated (Feng 2011; Zakeri 2009), at the time of writing the former group have not been analyzed in similar detail (Amambua-Ngwa 2012).

Population dynamics

The infectivity of a gametocyte carrier to the mosquito vector is controlled by numerous and diverse factors in the parasite (sex ratio, number, synchrony of asexual blood stages), host (antibodies, cytokines, and other plasma-derived inhibitors), vector (genetic susceptibility, midgut microbiota, concomitant infections) and the environment (see section above).

In a perfect world it would require just a single fertile male gamete to meet a single fertile female gamete in the 2- to 3-μL blood meal for infection to ensue. In the case of *P. falciparum*, where the female-to-male ratio is 3:1–5:1, this requires about three gametocytes per 1 μL of blood in a 2-μL blood meal. Persons with this density are well below the threshold of routine microscopy (20–50/μL), a concentration that some reports suggest marks a threshold for higher mosquito infection prevalence (Bousema and Drakeley 2011). Nonetheless, successful transmission has been described below this threshold (Bousema and Drakeley 2011). It has been suggested that 80% of all hosts infected with *Plasmodium* have gametocytes in their blood (Sinden 2012b); thus, the almost perennial question as to who represents the infectious reservoir is most conservatively stated as "Everyone who is infected." However, this statement is exactly incorrect. Although the interested researcher examining the biology of transmission might justify the cost of determining exactly who is infectious, an elimination campaign might consider it more cost effective to treat all infected persons.

Parasite dynamics in the mosquito

Whereas the molecular dissection and consequent reconstruction of the vital progression of *Plasmodium* in the mosquito continues apace, it is perhaps in the understanding of the parasite's population structure that key analyses have been made. Simply stated, we could not (and still cannot) answer the question, "How many new cases of malaria will result from mosquitoes feeding on this, or that, gametocyte carrier?"

Early studies summarized by Carter and Graves (1988) illustrated in *P. falciparum* the sigmoid relationship between log-gametocytemia and the prevalence of oocyst infection in the mosquito, a relationship subsequently confirmed (Churcher 2012) and replicated in *P. berghei* (Medley 1993; Churcher 2012). The later studies also identified the increasingly negative binomial frequency distribution of oocyst number as mean infection intensities fell.

Elegant life-table analyses (Vaughan 2006; Vaughan 1991; Vaughan 1992; Zollner 2005; Poudel 2008; Alavi 2003) identified very significant reductions in parasite number, in all parasite–vector

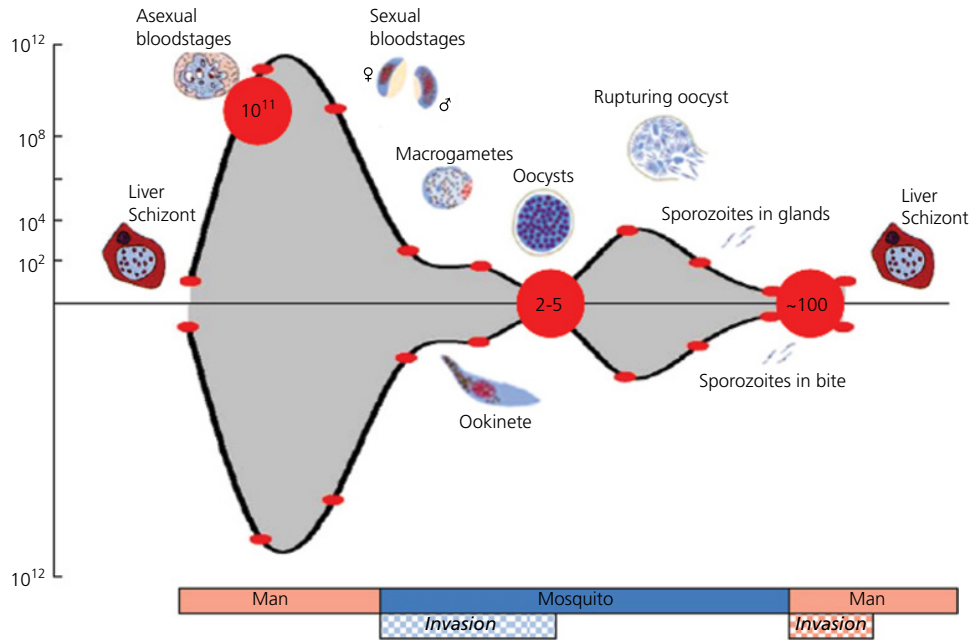

Figure 4.16 Graph illustrating the two key population bottlenecks through which malaria parasites pass as they are transmitted from the vertebrate to the mosquito, and from the mosquito back into the vertebrate host. Note parasite abundance is plotted on a log scale. (Image from Sinden, 2010.)

combinations studied, as the development progresses from gametocyte to ookinete- to oocyst, this particular sequence of transitions constituting the major bottleneck in the entire life cycle (Figure 4.16) (Sinden 2010). The influence of this contraction upon the genetic diversity of the parasites in the field is only now being brought to light (Chang 2012).

Examining the dynamics of sporogonic development across a range of parasite densities, it was shown that development from gametocyte–gamete, gamete–ookinete, ookinete–oocyst, and oocyst–salivary gland sporozoite are all related by saturating relationships, of which just the ookinete–oocyst transition illustrates an initial rising efficacy at very low ookinete numbers (Figure 4.17), and that particular relationship is sigmoid. Thus the salivary gland sporozoite number is not linearly related to the macrogametocyte number ingested or even to oocyst number. It is currently only in *P. berghei*, in which the dynamic relationships between all the successive intravector parasite stages have been defined experimentally, where this relationship can be computed (Figure 4.18) (Sinden 2012a).

The knowledge of these density-dependent relationships requires modification of the original Ross–MacDonald model (equation 1) to reflect more accurately on the component variables (equation 2), where (d) represents the density dependences of individual components.

$$R_0 = \frac{V}{H}\frac{a^2 b(d) c e^{-\mu_V(d)n}}{r\mu_V(d)} \qquad (2)$$

The importance of this analysis lies in the need to manage expectations when implementing transmission-blocking interventions. Implementation of an intervention reducing, for example, oocyst burden in the vector by 50%, at high transmission intensities will not reduce R_0 by 50%. However, repeated application of the measure may increasingly affect R_0 until elimination can be achieved (Blagborough 2013).

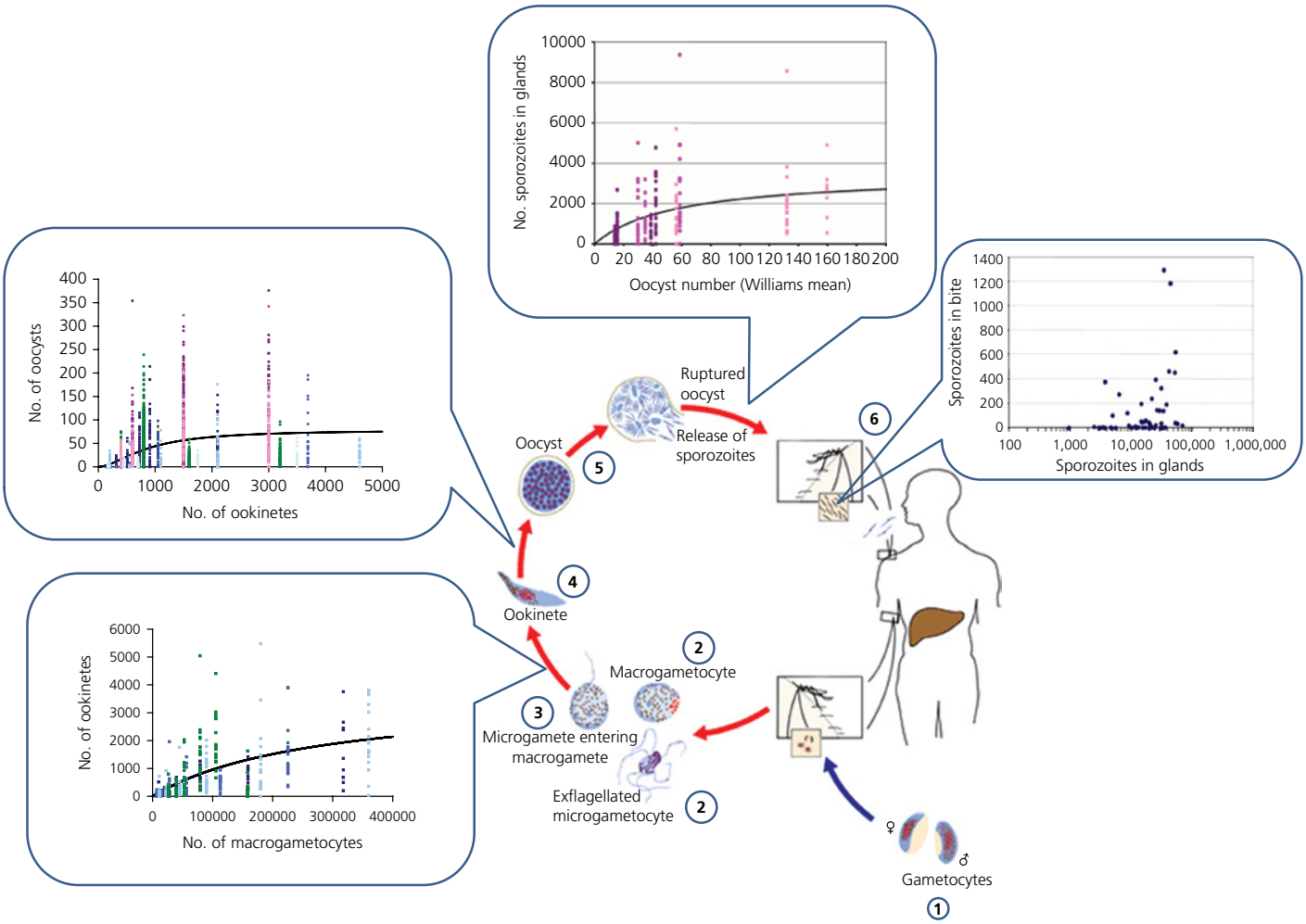

Figure 4.17 Diagram illustrating the density dependence observed in successive developmental transitions as *Plasmodium berghei* progresses through the mosquito *Anopheles stephensi*. 1, gametocytes; 2 and 3, gametes; 4, ookinete; 5, oocyst; 6, salivary gland sporozoites. (Image from Sinden *et al.*, 2008.)

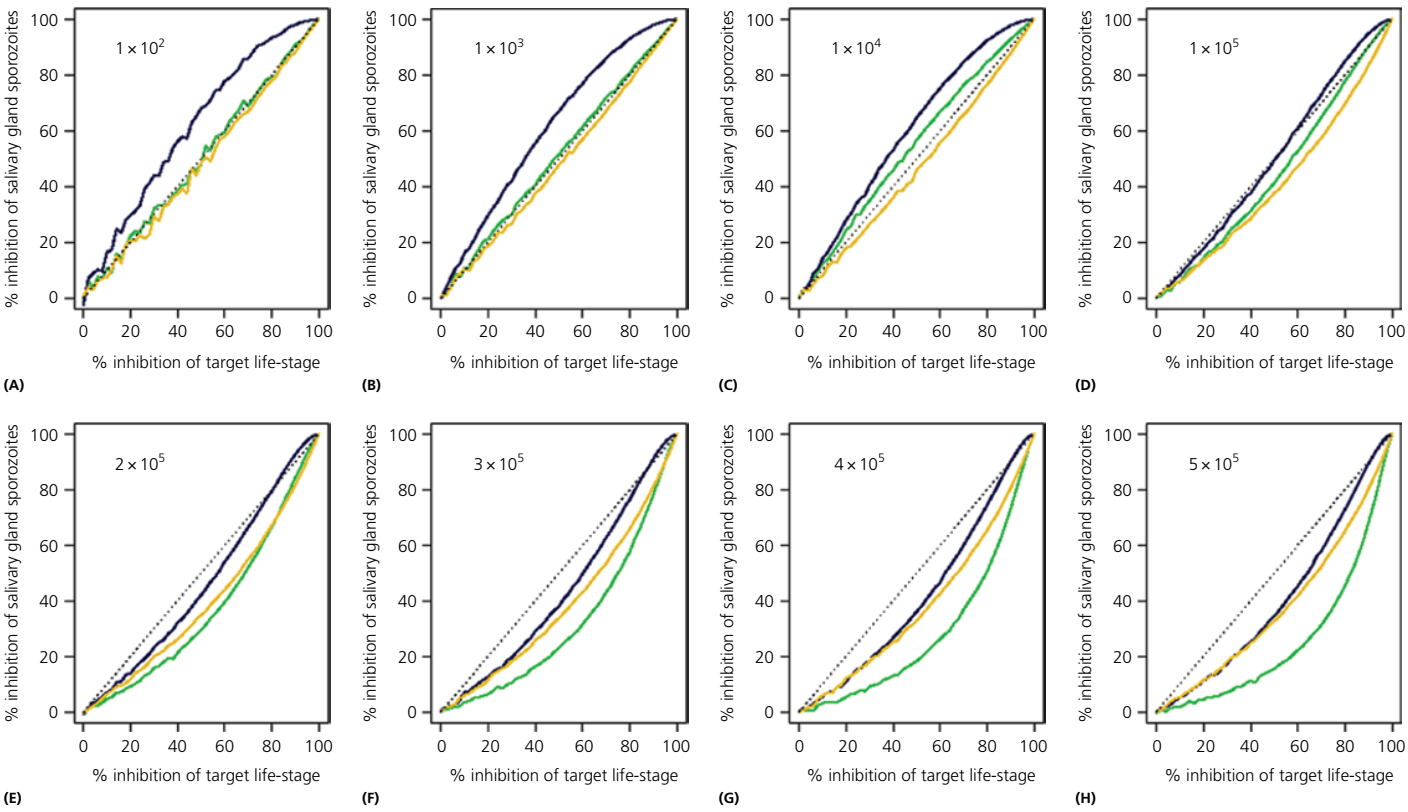

Figure 4.18 The theoretical relationships between the reductions that could be achieved by a transmission blocking intervention in oocyst (yellow), ookinete (blue), or macrogametocyte (green) numbers and the corresponding reductions that would ensue in salivary gland sporozoite number. Note how this relationship is markedly affected by the initial challenge infection (described as macrogametocyte number, top left corner of each box A–H) offered to the mosquito. (Image from Sinden, 2010.) (*See insert for color representation of this figure*.)

Despite this increase in understanding, a key relationship – that between salivary gland sporozoite burden and the probability of infection of the next host bitten – remains unresolved. There is evidence that very few (≤5) mosquito-delivered sporozoites may be required to infect a vertebrate host; indeed, Walliker (1983) reported that inoculation of a single sporozoite of *P. chabaudi* infected 1 of 93 mice. Laboratory studies on a wide variety of parasite and host species (including *P. vivax* and humans), taken together, suggest that 10 sporozoites, when injected by syringe, result in the infection of the host (Sinden 2008). Limited numbers of technically demanding studies have attempted to measure the number of sporozoites injected by an infected mosquito (Medica and Sinnis 2005; Kebaier 2009; Hellmann 2011; Ponnudurai 1991; Amino 2005). The most-detailed of these studies suggested that a mean of 123 (median 18) sporozoites are commonly delivered (range, 0–1297) and that the number injected is only weakly correlated ($R^2 = 0.12$) to the salivary gland burden. The mechanistic explanation for this may be that it is only those sporozoites already lying in the salivary duct that are delivered. It has been hypothesized that sporozoites accumulate in the duct to a spatially limited capacity (Figure 4.15) in the time between blood meals and are then ejected in the initial saliva droplet (Frischknecht 2006; Hillyer 2007a; Amino 2005). A logical consequence would be that it is the number of infectious bites that can be delivered by an infected mosquito will correlate more closely with gland burden. Boyd (1949) demonstrated that whereas a *P. vivax*–infected mosquito remained infectious for 40 to 56 days, the probability of its infecting the host fell over time. Whether this is due to the loss of sporozoites in successive bites or sugar feeds (Billingsley 1991), or to the senescence of the salivary gland parasite, was not resolved.

Although current evidence suggests every sporozoite-infected mosquito may logically be regarded as being infectious for the remainder of its life, we do not yet have any understanding of the mathematical relationship between salivary gland burden and the number of infections resulting. Not least of the moderating factors is the impact of the infection on the lifespan of the mosquito (Dawes 2009) and hence the number of blood meals taken by an infectious mosquito. Carefully designed studies to establish the burden–infection relationship should be a priority.

In understanding transmission in field settings, it is necessary to consider the many published observations as to how the intensity of malaria infections can compromise the fecundity (Hogg and Hurd 1995; Ahmed 1999; Rossignol 1986a), the flight performance (Rowland and Boersma 1988), and the ability of mosquitoes to transmit the sporozoites in the bite (Rossignol 1984a; Rossignol 1986b). The latter could be influenced by the reported reductions in body mass and in olfaction (hence the ability to find a blood meal) or by the ability to take further blood feeds (Rossignol 1986a; Dawes 2009). It is advisable that more data be collected in these areas to permit well-founded calculations on any impact that reducing the parasite burden in the mosquito population might have on transmission of the parasite over time within endemic populations.

Transmission-blocking interventions

Anti-vector strategies

When looking at the Ross–MacDonald formula (equation 1 or 2), it is obvious that measures directly attacking the vector population will have significant impact. The exponential terms a and p (equation 1) are clearly the most influential in determining impact upon R_0, the success of transmission. Thus, reducing the biting rate and survival of (infected) mosquitoes must remain priorities in this endeavour. The current armory of anti-vector measures is extremely diverse, including environmental, architectural, personal, and social management of human exposure to mosquito bite; to the environmental, house-based, bednet, and personal application of insecticides/biocides and/or repellents; the use of insect pathogens to enhance natural resistance to infection or to reduce mosquito

survival; the use of genetic modification techniques to reduce vectorial-capacity -fecundity, or -longevity; and possibly the use of vaccines that affect the susceptibility, fecundity, or longevity of the insect (Kebaier 2009). These are, however, comprehensively described in other reviews (e.g., Alphey 2014), and are not discussed here.

Anti-parasitic transmission-blocking interventions

Vaccines

In my view, transmission-blocking vaccines (TBVs) targeting the gamete and ookinete stages offer the best potential to contribute to the local elimination or eradication of *Plasmodium* species. Reasons for this optimism are founded on three pillars: First; the vaccines under development at the time of writing are showing, albeit in laboratory studies, substantial activity: suppressing *P. falciparum* oocyst number (intensity) by more than 95%. If these activities could be reproduced in populations in endemic areas, population studies (Blagborough 2013; Gething 2011) suggest these vaccines alone could eliminate the parasite if applied over multiple cycles of transmission. Second, current data suggest that if mechanisms can be found to reproduce in humans, the long-lived high-titer responses induced by Pfs25/OMPC in primates (Wu 2006), the response induced may persist long enough to achieve the repeated cycles of transmission reduction required to eliminate the parasite from a population (point 1 above). Third, some of the priority TBV candidates (e.g., P25) are never naturally exposed to the human immune system. P25 represents one of the more highly conserved genes in the parasite genome, and as a consequence it might be anticipated that a single vaccine might have global utility. In a similar context it might be anticipated that transmission-blocking immunogens from the mosquito midgut, which are not expressed in the insect's saliva, such as APN1 (Mathias 2012), might be similarly universal vaccine candidates, and in this case the effector response transcends *Plasmodium* species, potentially providing a truly universal vaccine candidate.

The current repertoire of TBV candidates is, however, worryingly limited. In this era of high-throughput genomics, transcriptomics, and proteomics (Wass 2012; Lasonder 2008; Patra 2008; Le Roch2003), it is a source of constant frustration to me that previous limited screens, based upon monoclonal antibody production, have not been expanded to review in greater depth the full repertoire of candidate vaccine targets. The limited early studies have identified three major classes of TBV target: the surface of the male and female gametes (e.g., P45/48; P230; HAP2), the surface of the ookinete (e.g., P25, P28), and mosquito midgut proteins (e.g., APN1) that bind the ookinete. Of these, P25, P230, and PHAP2 have shown the ability to reduce oocyst burden by more than 97% in a single cycle of mosquito infection (Miura 2012). It is perplexing that the high levels of transmission-blocking immunity described for APN1 (Mathias 2012) have not been replicated by others (Miura 2012; Kapulu in press), and the underlying reasons for this disparity requires investigation.

Drugs

Transmission-blocking drugs have, until very recently, not been a priority area of investigation. Nonetheless, in the 1950s, a substantial body of work demonstrated the ability of the sulfa drugs to suppress oocyst infections in mosquito species (reviewed in Butcher 1997), and thereafter work by Coleman and colleagues (Coleman 1990; Coleman 1992; Coleman 1994; Coleman 1988) further developed this knowledge base, but no significant product development ensued. The advent of artemisinin and the observation that treatment with this drug could reduce parasite transmission (Okell 2008) raised interest in this important group of therapeutic compounds but failed to engender the broader search for new transmission-blocking compounds. However, following the logical call to the research community to re-engage with the concepts of local elimination, and the call for global eradication of malaria parasites (Alonso 2011), the resultant expansion of studies seeking new drug targets in the gametocyte (Sáenz 2012; Lelièvre 2012; Adjalley 2011b; Buchholz 2011; Chevalley

2010; Lucantoni and Avery, 2012), gamete, ookinete, and oocyst (Delves2010; Delves 2012a,b; Delves 2012) has recently been revived.

To date, the prime effort has been to understand which of the current schizonticides have additional transmission-blocking activity, and numerous compounds with such properties have been identified (Figure 4.19). Although this duality of action may permit the rational prioritization of the current portfolio of schizonticides, it is, in my opinion, not the ideal route to follow simply because schizonticides must target the largest population of parasites found during the life cycle (~10^{11} in a patient with peak parasitemia) (Figure 14.16), and the rapid emergence of resistance to this class of compounds has previously compromised their useful life. By contrast, a drug exclusively targeting the mature gametocyte, gamete, ookinete, and oocyst would target about 10^9, 10^6, 10^3 or 10^1 cells respectively. It is surely the case that all else being equal, the speed of emergence of resistance will be strongly influenced by the size of population exposed. I would therefore advocate combining any new schizonticide with a second drug specifically targeting the smallest

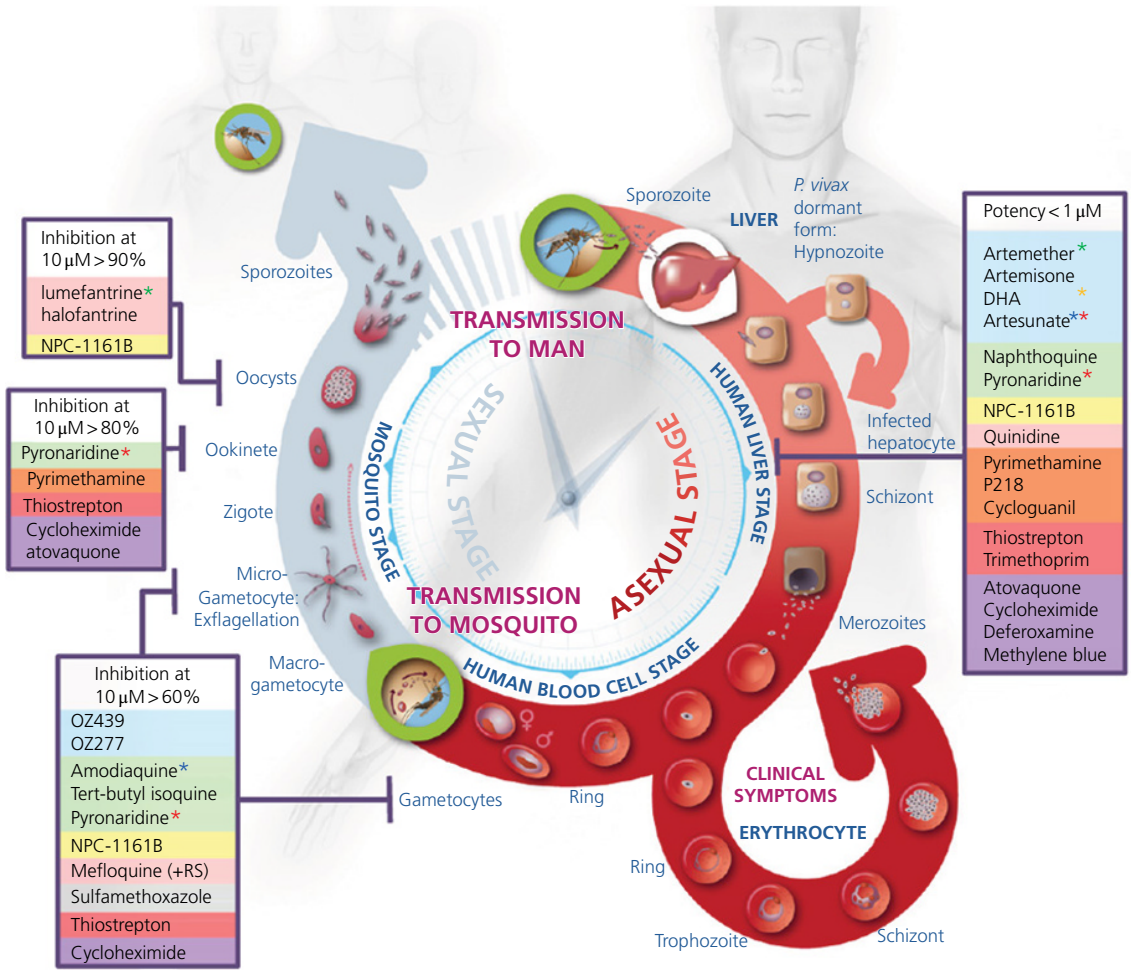

Figure 4.19 The activities of the most widely used current antimalarial compounds throughout the life cycle of *Plasmodium*. Stars denote components of current artemisinin combination therapies. Data obtained using *P. falciparum, P. yoelii,* or *P. berghei.* (Image from Delves *et al.*, 2012a.)

accessible population of parasites, which, recognizing the short half-life of most antimalarials at present, would be the gametes. It would be unforgivable if, once again we were diverted from a rational long-term decision by a need to gather the low-hanging fruit of an accessible but ill-found objective such as a drug that kills the developing immature gametocyte (but that leaves the mature gametocyte infectious to the vector).

Paratransgenesis

Ingenious lateral-thinking has postulated that genetic modification of commensals or symbionts such that the bacteria or fungi express molecules that directly, or indirectly, suppress malaria transmission might provide a novel means of intervention in the field (Dinparast 2011; Rasgon 2011; Wang 2012). Laboratory studies have achieved significant reductions in controlled populations (Bisi and Lampe 2011). However, it must be recognized that in the field there would be no possibility to control the selective pressure (inhibitor concentration expressed) exerted by an uncontrollable transgenic population. As with uncontrolled drug delivery, we must ask whether this interesting concept will prove to be evolutionarily sustainable in the field.

Determining the efficacy of transmission blockade

Recognizing that transmission-blocking interventions aim to reduce, over time, the number of new malaria cases in a population, it is perhaps surprising that at the time of writing all published assays to monitor the impact of transmission-blocking interventions fail to record these data but instead describe intermediate endpoints that have no established linkage to affect the vertebrate population (van der Kolk 2005; Churcher 2012). Since the time of Ross, the most common experimental design has been to observe the comparative reduction in the number of oocysts in replicated mosquito populations fed on aliquots of the same cultured (or host-derived) gametocytes exposed to experimental versus control interventions (van der Kolk 2005). Metaanalyses of this method have revealed the critical importance of reporting both the control infection intensity and the number of mosquitoes observed upon the validity of the blockade value reported (Churcher 2012). Other analyses have also described the comparative insensitivity of the membrane-feeding assay compared to the direct skin-feed assay (Bousema 2012), while simultaneously recognizing the eminent utility of the latter in that multiple, and comparative, observations can be made on gametocytes from a single source.

With the rediscovery of the need to have transmission-blocking drugs in our portfolio, a wide range of assays have been developed to screen for such compounds, in which the endpoints vary between gametocyte production (Adjalley 2011a), male gamete formation (Delves 2012b), female gamete formation (Delves, unpublished), ookinete production (Delves2012), and oocyst production (Delves and Sinden 2010). The relationships among gametocyte, ookinete, and oocyst endpoints, with perhaps the most important endpoint being salivary gland sporozoite burden, has been projected to differ significantly between the assays and with the challenge intensity (Figure 4.18) (Sinden 2012a). These data again highlight the inadequacy of our understanding of either the relationship between the salivary gland sporozoite burden and the probability any bite will be infectious, or with the lifetime potential of mosquitoes with different burdens to infect multiple hosts. Without these data, our understanding of the dynamics of transmission remains incomplete.

In an effort to overcome these inadequacies in the assays described above, a laboratory population, multi-cycle assay has been developed in *P. berghei* (Blagborough 2013). Using this method to study the impact of a transmission-blocking drug, atovaquone (which kills ookinetes with an IC_{50} of 5 nM), it was shown that this compound will reduce oocyst number in the standard direct feeding of mosquitoes on infected and treated hosts by 60% in one generation, and it could eliminate the parasite from the population in three transmission cycles, but only when the mosquito biting rate

was fewer than three bites per mouse per cycle. Importantly, the study shows for the first time that an anti-parasite transmission-blocking intervention can eliminate *Plasmodium* from a host population, but it simultaneously shows that we have a great deal more data to gather if we are to design and implement new transmission-blocking strategies based upon the huge riches of data that we now have at our command.

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CHAPTER 5

Comparative and functional genomics of malaria parasites

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Genomics is the study of the genome of a cell or an organism, including efforts to determine the entire DNA sequence of an organism and fine-scale genetic mapping. The essential goals of this field are to examine how an organism's genome sequence or structure dictates its phenotypes and responses to stimuli and to reveal its evolutionary history and relationships to other organisms. The field of genomics, including the genomics of malaria parasites, is growing quickly as new methods and tools are introduced every year. The discipline now comprises many subfields, with comparative, evolutionary, and functional genomics among the most active.

Comparative genomics is the study of diversity and divergence among the genomes of individuals (population genomics) or among species to reveal evolutionary history of a taxonomic group and to discover features unique to a particular lineage or species. Functional genomics may include studies of gene functions and gene expression regulation, including genome-wide analyses of mRNA and non-coding RNA (transcriptomics), protein expression (proteomics), products of cellular metabolism (metabolomics), DNA methylation and histone modification (epigenomics), interaction of an individual's genome with drugs (pharmacogenomics), and genome-wide association studies (GWAS), or linkage analysis to identify genetic variations linked to biologic traits or phenotypes. In this chapter, we summarize some of the major recent advances in the genomics of malaria parasites, with emphasis on comparative studies and functional genomics of the malaria parasite *Plasmodium falciparum*.

An Introduction to *Plasmodium* genomes

The genus *Plasmodium* is a diverse taxonomic group that includes malaria parasites of birds, lizards, rodents, and nonhuman primates in addition to the four parasites that naturally infect humans (Figure 5.1). *Plasmodium knowlesi* has been shown to be a zoonotic infection; however, humans are not a native host (Singh 2004). These parasites break down into three large groups: those of birds and lizards, the *Laverania* group of ape parasites (which includes *P. falciparum*), and a group of primate and rodent parasites that can be further divided between these two sets of hosts, with the first including many human parasites (*Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*) (Liu 2010; Martinsen 2008; Perkins 2002). Organisms with extensive genomic information available are either virulent human parasites (*P. vivax* and *P. falciparum*), parasites of rodents (*Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yoelii*), or those of monkeys and apes

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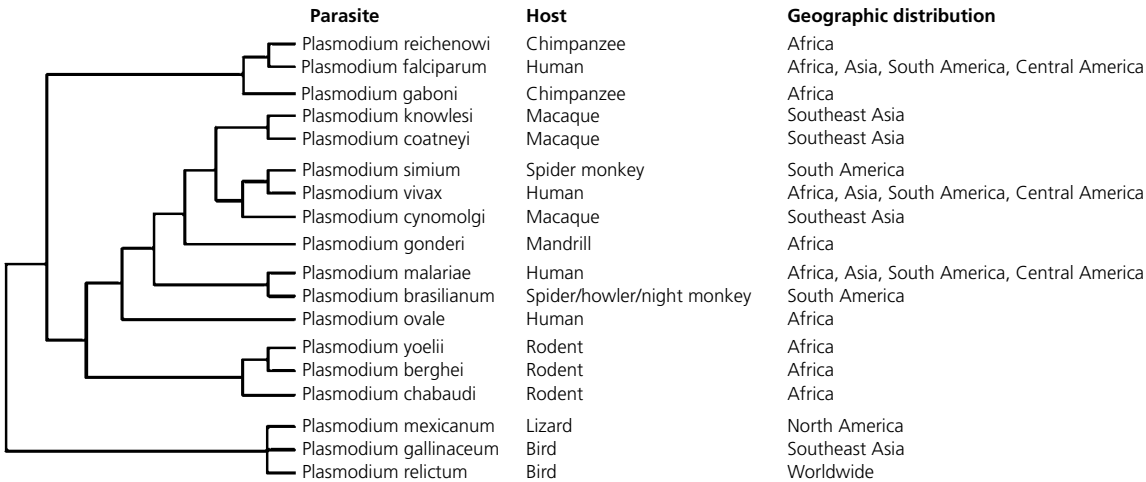

Figure 5.1 Cladogram showing relationships among *Plasmodium* parasite species based on information from Perkins 2002, Martinsen 2008, and Liu 2010.

Table 5.1 Genome data of some representative *Plasmodium* parasites.

Parasite	Natural Host	Size (Mb)	Coding (%)	Number of Genes*	A+T Content (%)			Source
					Exons	Intergenic	Overall	
P. falciparum	Human	23.3	52.6	5403	76.3	86.3	80.6	Gardner *et al.* 2002 Carlton *et al.* 2008
P. vivax	Human	26.8	48.5	5433	53.5	57.2	57.7	Carlton *et al.* 2008 Pain *et al.* 2008
P. cynomolgi	Macaque	26.2	51.0	5722	—	—	59.5	Tachibana *et al.* 2012
P. knowlesi	Macaque	23.5	47.4	5188	59.8	65.4	62.5	Carlton *et al.* 2008 Pain *et al.* 2008
P. yoelii	Rodent	23.1	50.6	5878	75.2	79.3	77.4	Carlton *et al.* 2002, 2008

*Numbers of predicted genes.

(*Plasmodium reichenowi*, *Plasmodium cynomolgi*, and *Plasmodium knowlesi*) that have been adapted to grow in laboratory animals *in vivo* (Table 5.1). Understanding the relatedness of these different parasites allows us to translate the findings of research based on one species to those of another. The emphasis on parasites directly involved in human health creates a somewhat uneven body of work on malaria genomics. Fortunately, advancement in genomics techniques may soon allow filling in the gaps.

The advent of the modern genomic era has changed the way we understand, study, and treat malaria as a disease. The first complete genome sequence for a malaria parasite was the genome of *P. falciparum* isolate 3D7 (Gardner 2002); however, predating the availability of genome sequences, scientists had made significant progress in understanding the basic structure of *Plasmodium* genomes

through pulsed-field gel electrophoresis and restriction fragment-length polymorphisms (Corcoran 1986; Freitas-Junior 2000; Ponzi 1990; Walker-Jonah 1992). We have gone from an early idea that malaria largely co-evolved with vertebrate hosts, echoing their evolutionary trajectory, to the understanding that these parasites have switched between hosts many times (Escalante 2005; Mu 2005b; Perkins 2002). We have also come to appreciate that many genomic features, such as high AT content and multigene families associated with virulence, have evolved multiple times in the lineage and that host switching is common over evolutionary time in *Plasmodium*. Genomic work has also pointed out the impact of recombination on short- and long-term molecular evolution in malaria parasites, enhancing our understanding of immune evasion, the evolution of virulence, and the evolution of the genome structure itself.

Genome structure of malaria parasites

The structure of a nuclear genome is defined by a number of characteristics. These features are used to facilitate comparative studies, allowing us to understand the organization of genetic material and indicating a great deal about the functional genetics of an organism. For nuclear genomes, the important structural elements are overall genome size, the number of chromosomes (karyotype) and whether this number is stable, the average base content (usually as a percentage of A+T or G+C), structural repeat sequences, and differences in genetic organization comparing central and subtelomeric regions.

Base content

The genomes of *Plasmodium* species have very different base content, with *P. falciparum* among the highest A+T content found in eukaryotes (an average of 80.6%, but 86.3% in non-coding sequence), whereas the sequences of many primate malaria parasites (*P. vivax, P. knowlesi*, and *P. cynomolgi*) have a balanced genome (50%–60% A+T), which is common among eukaryotes (Carlton 2008; Pain 2008; Tachibana 2012). Rodent malaria parasites (*P. berghei* and *P. yoelii*) are intermediate, with moderately high A+T content (about 77%) (Carlton 2002; Hall 2005) (Table 5.1).

Perhaps the first true comparative genomics study of *Plasmodium* examined the base content of seven parasites from monkeys (*P. knowlesi*, *Plasmodium fragile*, *P. cynomolgi*), humans (*P. falciparum*, *P. vivax*), birds (*Plasmodium lophurae*), and rodents (*P. berghei*) (McCutchan 1984). The study used cesium chloride density fractionation of whole-genome DNA to categorize the A+T base content: *P. berghei*, *P. lophurae*, and *P. falciparum* were categorized as high in A+T content, whereas *P. vivax, P. cynomolgi*, *P. knowlesi,* and *P. fragile* had relatively low A+T content. The results of A+T content have been confirmed by whole-genome sequencing (Carlton 2008, 2002; Gardner 2002; Hall 2005; Pain 2008; Tachibana, 2012) (Table 5.1); however, the evolutionary conclusion of the paper – that *P. falciparum* is more closely related to bird parasites than those of other primates – has not been borne out in later studies. The high A+T content appears as a common feature in the *Laverania* group of *P. falciparum,* rodent parasites, and parasites of birds, but the three parasite groups are only distantly related to one another (Figure 5.1) (Martinsen 2008; Perkins 2002).

This nucleotide difference has implications for codon usage/bias (DePristo 2006), evolution, and transcriptional mechanisms in the genome. Although all known eukaryote genomes show higher A+T content in noncoding sequences over coding (Oliver 1996), a 10% increase is notable and has implications for genome evolution. The resulting reduced complexity of the noncoding sequence contains large numbers of polyAs and polyTs that make it difficult to assemble sequence fragments; however, this repeat richness also generates an abundance of variable microsatellite loci that have been explored as genetic markers (Su 1996, 1999b).

Genome size and composition

Although the genomes of the two most virulent human parasites, *P. falciparum* and *P. vivax*, have been studied extensively for their importance to human health, a wealth of genomic information also exists on other parasites, allowing us to build a picture of the basic elements that comprise a *Plasmodium* genome. The genomes of malaria parasites are small compared with those of other eukaryotes, ranging from about 23.3 million base pairs in *P. falciparum* to an estimated 26.8 million base pairs in *P. vivax* (Carlton 2008) (Table 5.1). Evidence to date indicates that all *Plasmodium* genomes are about one-half coding sequence, with the noncoding content comprising intergenic sequence, telomeric repeats, introns, and noncoding RNAs (Carlton 2008; Hall 2005). There is no evidence for transposable elements in any *Plasmodium* species genome (DeBarry 2011). *Plasmodium* genomes generally contain more than 5000 genes, with counts ranging from 5188 in *P. knowlesi* to 5878 in *P. yoelii*. It should be noted that all these measures and counts are, to some degree, approximate because assembly and annotation of all these genomes are ongoing. The number of genes does not appear to correlate with genome size, and it varies among species (Table 5.1).

An estimated 80% to 85% of genes in *Plasmodium* genomes have orthologues (true homologues coming from sharing a common ancestor, as opposed to paralogues, whose homology comes from gene duplication) among all mammalian parasite species (Kooij 2005; Pain 2008). Of the genes with orthologues, the majority are located centrally on the chromosomes as opposed to in subtelomeric regions. The subtelomeric regions are locations for many species-specific gene families (discussed in more detail below) (Kooij 2005). Of all *Plasmodium* genes, about 50% still have no known function (Tachibana 2012).

Chromosome structure

Karyotypes of *Plasmodium* species remained obscure for some time because the chromosomes do not undergo a stage of condensation during division, when they can be easily visualized. With some effort, however, pulsed-field gel electrophoresis (Corcoran 1986) was made to show that the karyotype for all *Plasmodium* species is stable at 14 chromosomes, numbered from smallest (1) to largest (14).

The genes along the chromosome are not arranged in the same order when comparing species within the genus and are often not on the same chromosome. This observation means that chromosome 1 for one species is not necessarily truly homologous to chromosome 1 in another. The chromosomes of different species generally show synteny (physical colocalization of genetic loci on the same chromosome among species or individuals) of large chromosome blocks (Bozdech 2008; Carlton 2005, 1999), but synteny breaks down as compared species become more evolutionarily distant (DeBarry 2011; Kooij 2005). For example, the closely related species of *P. knowlesi* and *P. vivax* (Figure 5.1) show a single interchromosomal translocation over evolutionary time since their separation (Figure 5.2A), whereas *P. vivax* and *P. falciparum* – which are far more distantly related – show dozens (Figure 5.2A) (DeBarry 2011; Frech 2011).

Similarly, the closely related rodent malaria parasites *P. berghei* and *P. yoelii* (they were originally thought to be the same species (Killick-Kendrick 1978)) have no translocations, whereas the more distantly related *P. berghei* and *P. chabaudi* show three translocations (DeBarry 2011). Overall, the number of translocation events revealed among *Plasmodium* parasites appears to be low when compared with other eukaryotes (Kooij 2005), suggesting that this form of genome evolution is slow in these parasites.

It is important to note that translocation of chromosomal segments in *Plasmodium* have not occurred gene by gene but rather in large synteny blocks of sequence (DeBarry 2011; Kooij 2005). This allows us to see the modular evolution of genomes and that the unit of this evolution is not the

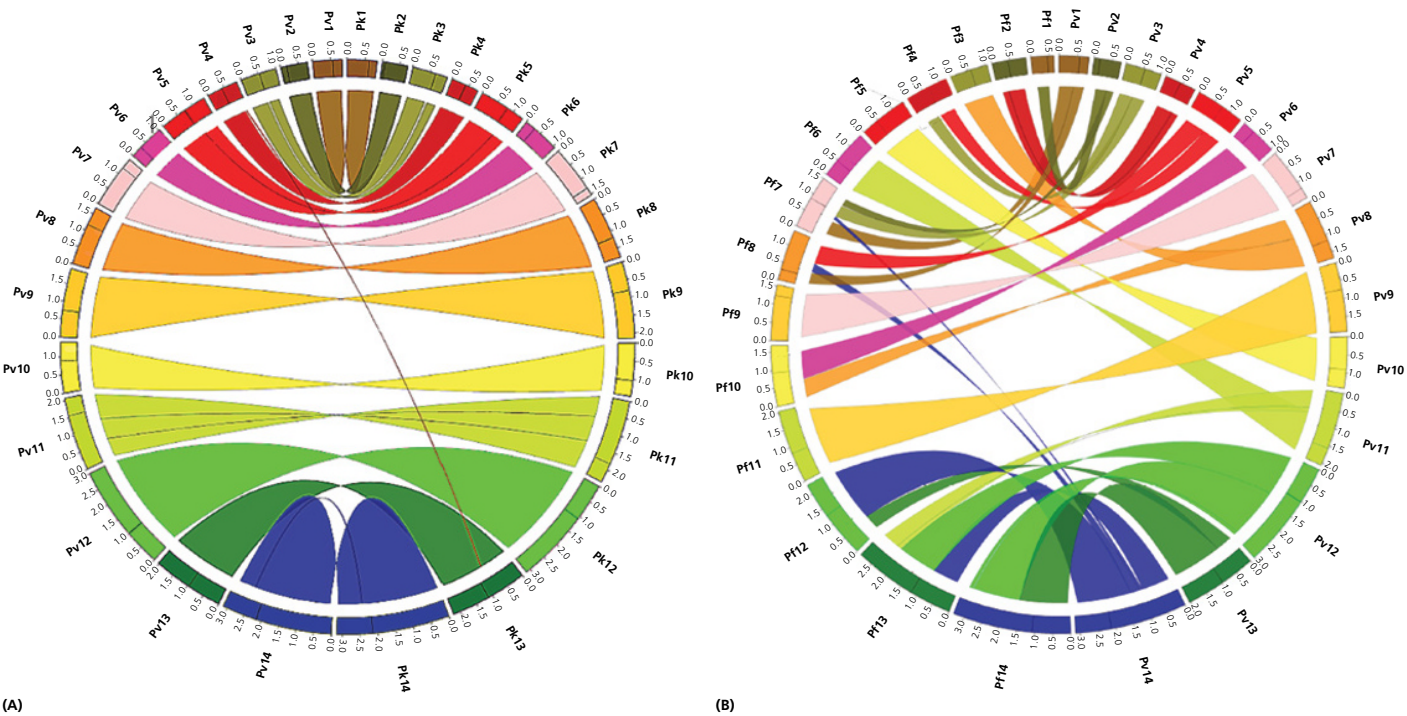

Figure 5.2 Synteny map of the *Plasmodium vivax* genomes as compared to *(A)* a closely related species, *Plasmodium knowlesi,* and *(B)* a more distantly related species, *Plasmodium falciparum*. Each block of color on the periphery of the circles represents a chromosome. Pk, *Plasmodium knowlesi*; Pv, *Plasmodium vivax;* Pf, *Plasmodium falciparum*. These figures show a larger number of rearrangements that have occurred over a greater amount of evolutionary time. They also show that the rearrangements do not occur as single genes, but as large "synteny blocks." (Adapted from Frech and Chen 2011.) (*See insert for color representation of this figure*.)

chromosomes *per se,* but blocks of sequence that are shuffled over time by recombination. For an example, *P. falciparum* chromosome 10, a relatively large chromosome, matches all of chromosome 6 in *P. vivax* (a relatively small chromosome) and half of chromosome 8 (Figure 5.2B). The other half of the *P. vivax* chromosome 8 matches with the small chromosome 3 of *P. falciparum* (DeBarry 2011; Frech 2011). These synteny blocks are often bounded by clusters of species-specific genes (described in more detail in the following section). These clusters of genes or gene families are scattered throughout genomes but are concentrated in the subtelomeric regions at the ends of the chromosomes. As a result, synteny breaks down frequently in the subtelomeric regions.

Although synteny blocks are a critical guideline for understanding *Plasmodium* genome structure, the other significant division within the chromosomes of malaria parasites is between the conserved central (centromeric) regions and highly diverse subtelomeric sequences, which are known to have elevated recombination rates and to frequently exchange genetic information both during meiosis (in the mosquito vector stage) and during mitosis (Foote 1989; Freitas-Junior 2000; Mefford 2002). Chromosome size is highly polymorphic in *Plasmodium,* with the majority of insertions and deletions in the subtelomeric regions (Corcoran 1986). These regions also contain many antigenic and species-specific genes and gene families. There are some notable exceptions to these rules, however: the Acyl-CoA synthetase genes that function as mitochondrial metabolic enzymes in most organisms are also located in the subtelomeric regions of *P. falciparum* (Bethke 2006). It is unclear whether the exception is due to these enzymes having landed in a zone of rapidly evolving loci or that they, in fact, have an important function in the parasite's virulence or immune evasion strategy.

Unlike the gene families in *P. falciparum*, members of the variant SICAvar antigen gene family in *P. knowlesi* are evenly distributed throughout the genome (Pain 2008). We can conclude from these observations that genes located toward the ends of a chromosome are likely under strong selection for increased diversity (such as genes coding for antigens) and are evolving rapidly, although the location of a gene in these regions does not directly indicate a function that necessitates this rapid evolution. Even with this caveat, careful annotation and assembly of the subtelomeric zones of the chromosomes – while challenging due to the amount of structural diversity located in these regions – is particularly important and informative.

Lineage-specific gene families

One of the most important applications of comparative genomics is identification of lineage-specific genes, most of which are in gene families (Tachibana 2012). *Plasmodium* parasites display many different phenotypes of interest to medical science: stimulation of inflammatory responses, immune evasion, cell invasion, relapses, recrudescence, virulence, and drug resistance. Each individual parasite, an isolate from a human patient or a lab-adapted strain, has a unique combination of these characters. Comparative genomics is often the first step in studying the origin of these differences and discovering the mechanisms of these features for the development of medical intervention.

Studying species-specific gene families is key to understanding the structure of *Plasmodium* genomes and telomeric versus central regions, because most lineage-specific gene families are located in the subtelomeric regions. These gene families are commonly expressed on the red blood cell surface and are thus highly diverse. This diversity is manifested in both gene copy number variation and gene sequences. These antigenic genes are often highly polymorphic, to the point at which they do not have a true allelic structure, as no loci are orthologous among isolates (i.e., they are all paralogous, because they do not have stable positions in the genome). It is important to note, however, that the regions of diversity within these genes are not distributed evenly throughout the sequence. Rather, the diversity in these genes is localized in confined sections of the genes where the level of sequence conservation among paralogues is low. These regions are bound by relatively

more conserved sequences. This distribution of the highly polymorphic regions is critical for predicting epitopes of immune response and for vaccine design, because regions of increased diversity are likely to be exposed to immune pressure from the host.

P. falciparum-Laverania

For *P. falciparum*, the critical species-specific gene families include the variant antigen genes (usually called the *var* genes) (Su 1995), which code for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), the repetitive interspersed family (*rif* genes, coding for RIFINS), and subtelomeric variable open-reading frame *(stevor)* genes (Cheng 1998; Gardner 2002). The *var* genes are a large family, with around 60 genes in each haploid genome and the number varying among isolates (Kraemer 2007; Su 1995) due to continuous gene duplications and deletions from unequal crossovers (Freitas-Junior 2000). The genes are spread throughout the genome and found either in tandem arrays internally or clustered in the subtelomeric regions associated with the rifins and stevors. There is much to learn about function and regulation of the gene families, but great progress has been made on the *var* genes since they were identified as a major virulence factor (Baruch 1995; Smith 1995; Su 1995). Studies have shown that *var* genes are not unique to *P. falciparum*; the closely related *P. recheinowi* (Figure 5.1) contains this gene family, as well (Bull 2008). Little detail is known about these lineage-specific genes outside of *P. falciparum*, but future genomic work will likely shed much light on these gene families.

The *var* genes are probably the most extensively studied gene family in *Plasmodium*, because the proteins for which they code—the PfEMP1s—are implicated in both virulence and immune evasion. These *var* genes are variable in size but have a standard two-exon structure with a small intron that contains regulatory sequence. The first exon is between 3.5 and 9.0 kb long and codes for the extracellular portion of the protein, comprising a series of Duffy binding-like (DBL) and cysteine-rich interdomain regions (CIDR). The second exon (1–1.5 kb) contains a sequence that is far more conserved and encodes a cytoplasmic tail that contains acidic amino-acid residues known as the acidic terminal sequence (Gardner 2002; Su 1995). The sequence diversity is focused in the first exon, with small regions (15–50 residues) of relative conservation interspersed with longer regions that are more divergent. A complex interplay of evolutionary mechanisms is responsible for generating this diversity, comprising non-allelic recombination, point mutation, deletion, and duplication (Bull 2008; Freitas-Junior 2000; Kraemer 2007).

This rapid evolution has also made understanding and categorizing the relationships of these paralogues difficult (Barry 2007; Lavstsen 2003). A number of categorization systems have been proposed (Lavstsen 2003; Rask 2010), but the large amounts of diverse sequences make classification of these genes difficult. PfEMP1s are expressed on the surface of the infected red blood cell and are thus one of the proteins directly exposed to the human immune system (Baruch 1995; Smith 1995). These proteins have adhesive properties and allow the red blood cells to adhere to the vascular endothelium, keeping late stages of the parasites out of the peripheral circulation and the reticuloendothelial system. This cytoadherance also provokes an inflammatory response and blocks the microvasculature, resulting in much of the pathology associated with falciparum malaria (Baratin 2007; Ochola 2011). In addition to their role in malaria pathology, the diverse and constantly changed epitopes also allow the parasite to evade the host immune system.

Other large variant families include the *rif* and *stevor* genes – which also have two-exon structure – that are found in subtelomeric regions closely associated with *var* genes. The *rif* genes are the largest gene family in the Laverania lineage, with an estimated 150 to 200 copies per isolate (Cheng 1998; Gardner 2002), and can be separated into two subfamilies, A and B, with the first group containing about three quarters of the genes and being slightly larger than the remaining genes due to a 75-bp insertion (Joannin 2008). The small first exon codes for a signal peptide (50–75 bp), and

the larger second exon (about 1 kb) contains a variable sequence with a *Plasmodium* export element (Pexel) motif and a variable domain (Cheng 1998). Bordering the variable region, which is exposed on the cell surface, are two transmembrane domains (Carlton 2002; Cheng 1998; Joannin 2008; Niang 2009). The *stevor* genes are fewer and less polymorphic, with only an estimated 30 to 40 paralogues in each genome (Blythe 2004; Cunningham 2010). The proteins encoded by these genes are expressed on the surface of the infected red blood cell, and although their exact functions remain obscure, they are clearly distinct and may play an important role in antigenic variation (Blythe 2004).

Non-Laverania mammal parasite lineages

Two major groups of lineage-specific gene families are found in the non-Laverania mammal parasite lineages, the *Plasmodium* interspersed repeats *(pir)* genes, of which each parasite has its own subgroup, and the schizont-infected cell agglutination *var* (SICAvar) genes. Although the functions of all these genes remain somewhat obscure, their discovery in almost every parasite – either directly by cloning or indirectly by studying the effects of their encoded proteins – resulted in a major step forward in our understanding of antigenic variation in malaria parasites (Al-Khedery 1999; Kyes 2001). The *pir* gene families are the best-characterized genes families in the non-Laverania parasites. Genes of this family are found in all non-Laveranian parasite genomes sequenced to date, such as *P. berghei (birs), P. chabaudi (cirs), P. cynomolgi (cyirs), P. knowlesi (kirs), P. vivax (virs),* and *P. yoelii* (*yirs*). Despite the similarities in naming, and some conjecture as to the similarities of the primary and secondary protein structures (Janssen 2002; Janssen 2004), inclusion of the *rif* genes of the Laverania group into this group is controversial (Cunningham 2010).

The *pir* genes were first described in *P. vivax* (del Portillo 2001), the *vir* genes, although they were indirectly detected as early as 1962 in *P. berghei* when the first evidence of antigenic variation was discovered (Janssen 2002). They generally have a three-exon structure, although the numbers of exons can vary among species (Cunningham 2010). A typical gene has a small first exon followed by a large second exon that usually accounts for 80% of the coding region, and then a small third exon (del Portillo 2001; Janssen 2002). The presence or absence of transmembrane domains is also variable in each gene family (Cunningham 2010; del Portillo 2001). Although extensive attempts have been made to cluster or otherwise classify the species-specific gene families into an organized system, all attempts leave a notable number of the genes uncategorized (Carlton 2008; Cunningham 2010; Janssen 2004). The number of *pir* genes in a genome varies greatly, with *P. knowlesi* having 70 *kirs* genes and *P. yoelii* having 838 *yirs* genes (Carlton 2002; Kooij 2006; Tachibana 2012). Unlike the *var* genes, not all *pir* genes are localized in subtelomeric regions (Cunningham 2010). Due to differences in the size of the paralogues and differing numbers in the families, it is difficult to assess and compare the overall levels of divergence within and among the gene families. The *vir* genes are notably more divergent among each other than the *birs, cirs,* or *yirs* at the sequence-level; however, the rodent-lineage *pir* families appear to be evolving more quickly in terms of the structure of the families themselves, with many duplication and deletion events shaping the size of the gene families and their groupings (Figure 5.3) (Cunningham 2010).

The SICAvar gene family of *P. knowlesi* is one of the most interesting within *Plasmodium*. The phenomenon of antigenic variation maintaining infection in malaria was revealed in *P. knowlesi*, leading to the discovery of these genes (Brown 1965). To date there are 242 predicted SICAvar genes (including pseudogenes and fragments, which predominate) (Tachibana 2012) that are evenly distributed throughout the genome (Calafell 2008). The genes have a complex structure, ranging between 3 and 14 exons, with multiple exons coding for a series of cysteine-rich domains followed by a transmembrane domain and a cytoplasmic domain (Pain 2008). Despite their name, expression pattern, and gene structure similarities, the SICAvar genes do not have any sequence homology or common ancestry with the *P. falciparum var* genes (Al-Khedery 1999).

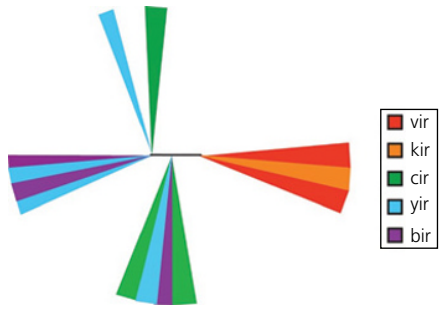

Figure 5.3 Evolutionary relatedness of genes of the *pir* families. Each wedge represents a group of paralogues from each different subfamily of *pir* genes. Note that the primate parasite genes group together, the *virs* (from *P. vivax*) and *kirs* (from *P. knowlesi*) group separately from the rodent parasite genes; however, they do not form a single group by species. Similarly, some of the rodent *pirs* group by species relatedness (a group of *yirs* from *P. yoelii* and *birs* from *P. berghei*), shown in Figure 5.1. Other rodent *pirs* group all together. This shows that the defining characteristics of the rodent and primate *pir* paralogues originated after the split of these two larger groups (see Figure 5.1), whereas within the groups many paralogues retain older characteristics from before the species split. (*See insert for color representation of this figure.*)

From genome sequences to gene function

Understanding the structure and content of malaria parasite genomes is the starting point for examining how genomic elements interact and function. The goal of these studies is to uncover the characteristics of the parasites that allow us to pinpoint weaknesses and thus identify effective preventive and therapeutic strategies. A gene's function can be reflected in the phenotypes it associates with, and phenotypic variation can then be linked to changes in a functional domain or expression level of the gene. Development of large-scale genome-wide functional tools and methods (e.g., microarrays, next-generation sequencing, quantitative mass spectrometry [MS], chromatin-immunoprecipitation, computational software, high-throughput drug screening) has been used to mine *Plasmodium* genomes in an unbiased manner and to identify genetic determinants that may be useful in the fight against malaria parasites. In the following section, we summarize major advances in some rapidly evolving fields of malaria functional genomics. It is not possible to include all the significant advances in the limited space here; we apologize for not being able to cite all the relevant studies.

Genetic mapping

Genetic mapping is a powerful tool that has been widely used to study genetic polymorphisms associated with different phenotypes of malaria parasites. There are two approaches: linkage mapping using progeny from a genetic cross and association analysis using parasites isolated from patients. Association mapping can be divided into candidate gene association and GWAS. The technique of crossing malaria parasites was initially developed using rodent malaria parasites (Walliker 1971) and was later extended to human malaria parasite *P. falciparum* (Walliker 1987). In brief, two strains of the same species of malaria parasites with distinct phenotypes of interest are crossed to generate recombinant progeny. Then individual recombinant progeny are isolated from a pool of uncloned products and are genotyped using genetic markers to determine parental alleles throughout the haploid genomes that are inherited through meiosis and sexual recombination following Mendelian principles. Following characterization of phenotypes of recombinant progeny, genetic loci that are linked to a specific phenotype can be identified and candidate genes characterized.

At present, three genetic crosses (3D7 × HB3, HB3 × Dd2, and 7G8 × GB4) have been conducted among five strains of the human malaria parasite *P. falciparum* (Hayton 2008; Walliker 1987; Wellems 1990). These crosses have been used to identify genetic loci or determinants linked to many biologically and medically important traits, including resistance to antimalarial drugs (Beez 2011; Fidock 2000; Peterson 1988; Sa 2009; Su 1997; Wang 1997; Wellems 1990), erythrocyte invasion and virulence (Hayton 2008; Wellems 1987), parasite sexual stage development (Furuya 2005; Vaidya 1995), nutrient transport (Nguitragool 2011), responses to a large number of chemicals (Yuan 2011; Yuan 2009), candidate pathways regulating cell-cycle duration (Reilly Ayala 2010), and genome-wide expression level polymorphisms (ELPs) (Gonzales 2008). Whereas a phenotype can be determined by a major gene, the majority of phenotypes, such as parasite responses to quinine, are complex traits controlled by multiple genes (quantitative trait loci, or QTL) (Ferdig 2004).

In addition, multiple genetic crosses using rodent malarias *P. yoelii* and *P. chabaudi* have also been performed, providing a platform to study host and parasite interaction and disease phenotypes that are difficult to study in humans. Classic genetic mapping in rodent malaria parasites has led to identification of the loci associated with resistance to chloroquine, sulfadoxine–pyrimethamine combination, and mefloquine in *P. chabaudi* (Carlton 1998; Cravo 2003; Hayton 2002; Hunt 2004) and of the loci linked to growth-related virulence phenotype in *P. yoelii* (Li 2011). Many loci identified in rodent malaria genomes are also present in human malaria parasites, suggesting that molecular pathways of drug responses are conserved across the species of *Plasmodium* parasites (Carlton 2001a).

In addition to the classic approach of cloning progeny from a genetic cross, an alternative method called linkage group selection (LGS) was developed in rodent malaria models (Carter 2007). In this method, the laborious procedure of cloning and characterizing recombinant progeny of a genetic cross is circumvented. Rather, the entire population of uncloned product of the genetic cross is placed under selection pressure that represents a biologic characteristic of interest, such as drug or immune selection. DNA of the selected progeny is screened *en masse* with quantitative parental clone-specific genetic markers across the genome. It would be expected that alleles of genes not linked to the locus affected by selection pressure would retain approximately the same frequency in the cross progeny before and after selection. By contrast, genetic markers of the parental clone sensitive to selection pressure located near the gene conferring susceptibility to the selection pressure will be removed or reduced in the cross progeny following selection. The level of reductions of sensitive alleles is proportional to the intensity and strength of selection pressure.

Using the LGS approach, many genetic loci or determinants of multiple complex traits of rodent malaria parasites have been located, including responses to antimalarial drugs such as pyrimethamine (Culleton 2005), mefloquine (Borges 2011), chloroquine (Kinga Modrzynska 2012), and artemisinin and artesunate (Hunt 2007, 2010); targets of strain-specific protective immune responses (Cheesman 2010; Martinelli 2005; Pattaradilokrat 2014); and blood-stage multiplication rate (Pattaradilokrat 2009). Functional analysis of candidate genes in rodent malaria parasites might also help interpret the functions of orthologues in human malaria parasites.

Although the genetic cross approach has been extremely useful in locating the regions and genes in the parasite genome linked to important traits, the procedures are labor intensive and expensive. The method for producing genetic crosses in human malaria parasites also requires the use of nonhuman primates, which is of great ethical concern. An alternative approach is GWAS, which was originally developed to identify disease genes in the human genome. A primary step in GWAS involves systematic characterization of global genetic variants in parasite populations. Large numbers of polymorphic sites in the *P. falciparum* genome have been identified (Jeffares 2007; Manske 2012; Mu 2007, 2002; Su 1999a; Volkman 2007). Nearly 200,000 single nucleotide polymorphisms

(SNPs) have been identified from sequenced *P. falciparum* field isolates (http://www.ncbi.nlm.nih.gov/projects/SNP/), including about 86,000 high-quality exonic SNPs from 227 samples from Africa, Asia, and Oceania (Manske 2012). With large numbers of SNPs characterized, various high-throughput genotyping microarray platforms have been developed for efficiently genotyping malaria parasites (Pattaradilokrat 2013). These SNPs and typing methods have been employed to detect nucleotide substitutions and copy number variation (CNV) in *P. falciparum* field isolates (Bozdech 2013; Cheeseman 2009; Daniels 2008; Dharia 2009; Jiang 2011; Jiang 2008; Kidgell 2006; Mackinnon 2009; Mu 2010; Neafsey 2008; Samarakoon 2011; Takala-Harrison 2013; Tan 2011). Subsequently, genotypes of individual parasites with different phenotypes of interest are compared so that a variant (allele) of a gene may be associated with a specific phenotype.

GWAS and linkage mapping each has its own advantages and disadvantages. Association using natural parasite populations can sample a spectrum of diversity of phenotypic determinants (important for multigenic traits) but requires assumptions of certain population structure and mating patterns. In contrast, the diversity of the two parents of a genetic cross is limited to what is contained in the parents when the classic genetic analysis is conducted. Some determinants may be missed, but genetic background noise is greatly reduced (Su 2004).

GWAS has been employed to study drug-related phenotypes using natural parasite populations (Cheeseman 2012; Mu 2010; Park 2012; Van Tyne 2011). Using a microarray chip based on Affymetrix molecular inversion probe (MIP) technology, Mu and colleagues genotyped DNA samples from 189 culture-adapted *P. falciparum* parasites from worldwide collection (Mu 2010). The genotypes were used to infer parasite population structure, to determine variation in recombination rate, and to detect loci under recent positive selection and genetic loci associated with parasite responses to multiple drugs (Mu 2010). In a similar study, Van Tyne *et al*. used a high-density genotyping array containing more than 17,000 SNPS to genotype 57 culture-adapted parasites from three continents and identified a locus associated with parasite responses to halofantrine, mefloquine, and lumefantrine (Van Tyne 2011). Overexpression of a candidate gene (PF10_0355) was found to make the parasite less sensitive to the three drugs.

More recently, a locus on chromosome 13 of *P. falciparum* was found to be under selection and associated with an artemisinin delay clearance phenotype after characterizing 6969 SNPs in 91 parasites from Cambodia, Thailand, and Laos using a custom-made microarray (Cheeseman 2012). Similarly, parasites collected from Bangladesh, Thailand, and two sites in western Cambodia were genotyped at 8079 SNPs using an upgraded MIP SNP array, and four SNPs on chromosomes 10 (1 locus), 13 (2 loci), and 14 (1 locus) were significantly associated with delayed parasite clearance (Takala-Harrison 2013). These studies suggest that GWAS can be applied to study malaria phenotypes, particularly those under selection; however, association studies will have to deal with noise from population structure, unknown selection forces, and, often, small parasite sample size; therefore, functional verification of candidate genes is necessary. Although the majority of genome-wide analyses were performed in *P. falciparum*, similar studies of *P. vivax* and *P. cynomolgi* using microarray and/or genome-wide sequencing have also been reported (Anfosso 2006; Dharia 2010; Neafsey 2012; Tachibana 2012).

One primary challenge in genetic studies of malaria parasites is the identification of mappable phenotypes. The malaria parasite is a small unicellular organism, and it is almost impossible to distinguish morphologic differences between parasite isolates under a microscope. The presence of large numbers of polymorphisms in the parasite genomes (Jeffares 2007; Manske 2012; Mu 2007; Volkman 2007) suggests potential variations in parasite physiology, development, and response to outside environmental stimulation. One strategy to display the potential differences is to characterize parasite responses to a large number of chemical compounds. Genetic loci associated with phenotypic variation between a pair of genetic cross parents or among a collection of field isolates

can be localized using linkage or association mapping, including GWAS. Indeed, large numbers of differential chemical phenotypes were identified and mapped to *P. falciparum* genes, particularly to the three genes known to contribute to drug resistance: *P. falciparum* dihydrofolate reductase or *pfdhfr, P. falciparum* multidrug resistant transporter-1 *or pfmdr1,* and *P. falciparum* chloroquine-resistance transporter, *pfcrt* (Yuan 2011; Yuan 2009).

Population genomics to identify vaccine or drug targets

Population genomics is the large-scale comparison of genomic DNA sequences within a population or between populations, which is an extension of traditional population genetics. The discipline deals mostly with allele frequency distribution and the dynamics of alleles in a population under the influence of different evolutionary processes such as natural selection, genetic drift, mutation, recombination, and gene flow. Genetic diversity of parasite populations can be used to infer parasite evolutionary history (Ayala 1999; Conway 2003, 2000b; Escalante 2005; Mu 2002, 2005b; Rich 1997; Verra 2000; Volkman 2001), population structure, and genetic recombination frequency (Conway 1999; Manske 2012; Mobegi 2012; Mu 2005a, 2010). It can also be used to identify genes under positive selection such as drug targets (Mu 2010; Takala-Harrison 2013) or genes under balancing selection such as immune targets (Amambua-Ngwa 2012a; Anderson 2004; Baum 2003; Conway 2000a; Polley 2001; Tetteh 2009).

One most likely application of population genomics and genetics for drug and vaccine development is identification of genes under various selection forces. Mutations in a drug target can be identified by searching for signature of directional selection or selective sweep (i.e., reduction or elimination of variation in genomic regions). Indeed, the *pfcrt* gene could have been identified using the approach of population genomics because of a strong drug-selection signature (Wootton 2002). The same principle has been applied to detect signatures of selection on other drug targets in human malaria parasites (Cheeseman 2012; Hawkins 2008; McCollum 2007; Mu 2010; Nair 2003; Roper 2003, 2004; Vinayak 2010). In contrast, most antigen-coding genes that are targeted by allele-specific protective immunity are subjected to balancing selection, leading to higher levels of genetic diversity in genes under host immune pressure. Search of genes with high levels of polymorphisms led to identification of some novel antigens that are recognized by immune sera from malaria-endemic populations and can be potential new malaria vaccine candidates (Mu 2007).

Applying next-generation high-throughput sequencing, Amambua-Ngwa and colleagues generated paired-end short-read sequences from 65 parasites in clinical isolates from an endemic Gambian population and found that 2853 genes contained three or more SNPs. From the data, a group of genes with signatures of selection were identified, including those highly expressed in the merozoite stage such as members of *clag*, PfMC-2TM, *surfin*, and *msp3*-like gene families (Amambua-Ngwa 2012b). Together, the polymorphic genes under balancing selection may be considered for functional study and for evaluation for vaccine development.

Global analysis of transcripts

Analysis of genome-wide mRNA transcripts can provide important information on gene function and gene expression regulation. Characterization of global genes transcribed at specific developmental stages or under a specific condition has been one of the major approaches employed to predict gene function. Collection of malaria mRNA transcripts on a large scale started with sequencing of expressed sequence tags (ESTs) in the early 1990s when the first generation of fluorescent DNA sequencer was introduced (Chakrabarti 1994; Reddy 1993). The main goal of these efforts was gene discovery, such as identifying the expressed genes in the parasites during different stages of development. For example, a gene discovery analysis of approximately 25,000 random genomic and ESTs from three species of *Plasmodium* (*Plasmodium berghei* ANKA, *P. vivax*, and *P. falciparum*) were

used to predict probable open-reading frames for proteins and for functional classification, providing a valuable resource for further studies on gene expression and gene function (Carlton 2001b).

Subsequently, serial analysis of gene expression was applied to collect genome-wide expression data (Munasinghe 2001; Patankar 2001). These studies identified about 4866 transcripts in an asynchronous blood stage of *P. falciparum*, including the antisense strand of annotated genes. The identification of antisense transcription suggests that antisense transcripts might play a role in regulating gene expression. More efforts followed to obtain full cDNA from *P. falciparum* (Lu 2007; Tuda 2011; Watanabe 2001, 2007). However, these efforts have not been as successful as those done in human or mouse due to high AT content in the noncoding regions.

With the advancement of technologies for immobilizing large numbers of nucleotide probes on a small glass slide, microarray analysis was quickly adopted for analysis of gene transcription in malaria parasites. In the first microarray study of malaria parasites, 3648 random inserts from a *P. falciparum* mung bean nuclease genomic library were used to construct a shotgun DNA microarray (Hayward 2000). Large differences in gene expression were identified between the blood-stage trophozoite and gametocytes, leading to lists of stage-specific transcripts. Completion of the *P. falciparum* genome project and publication of the parasite genome sequences in 2002 (Gardner 2002) allowed design and printing of various microarrays for studying gene expressions (Bozdech 2003a, 2003b; Le Roch 2002, 2003).

The first two groundbreaking studies using two different types of microarrays with genome-wide probes revealed interesting and important information on *P. falciparum* gene expression and regulation (Bodzech 2003b). Synthesized one or more 70-mer oligonucleotides selected from the 3′ end of each of about 6000 predicted genes (open reading frames) showed extensive transcriptional regulation of genes specialized for trophozoite and schizont stages of *P. falciparum* (HB3 isolate), suggesting extensive transcriptional regulation during parasite development (Bozdech 2003a). At about the same time, Le Roch *et al*., designed a custom Affymetrix array containing 260,596 25-nucleotide single-stranded probes from predicted coding sequences and 106,630 probes from noncoding sequences (Le Roch 2003) and used the array to detect transcripts from different developmental stages of *P. falciparum* (3D7 isolate). Clusters of stage-specifically expressed and/or regulated genes were characterized, providing a detailed transcriptional profile for different stages of the life cycle of the parasite. Despite differences in the parasite isolate and microarray technology (long oligonucleotides versus Affymetrix short oligonucleotides), both studies showed comparable expression patterns of the two parasite strains, indicating the conserved and coordinated transcriptional pathway in *P. falciparum*.

Various microarrays have been developed and used to investigate parasite responses to antimalarial drug treatment (Crameri 2007; Cui 2012; Dharia 2009; Ganesan 2008; Istvan 2011; Jiang 2008; Kritsiriwuthinan 2011; Mok 2011), expression of multigene families (Claessens 2012; Lawton 2012), gametocyte development (Eksi 2012; Young 2005), and parasite responses to starvation (Babbitt 2012; Fang 2004) and to identify regulatory elements that control gene expression in the parasite's genomes (Campbell 2010; De Silva 2008; Young 2008). Microarray was also employed to investigate gene expression patterns of parasites derived directly from patients' blood (Daily 2007; Mackinnon 2009; Siau 2007). More recently, DNA microarrays were used to show that *P. falciparum* parasites with delayed parasite clearance after artemisinin treatment had a marked decrease in metabolic activities in the ring stages, which might allow the parasite to survive oxidative stress induced by artemisinin (Mok 2011).

In addition to mRNA-related transcriptome, large numbers of short or long noncoding RNA (ncRNA) have been characterized using microarray (Broadbent 2011; Chakrabarti 2007; Li 2008; Mourier 2008; Raabe 2010). Noncoding RNA and/or antisense RNA are likely to play an important role in gene expression regulation and other diverse cellular processes of malaria parasites. In a

study using a high-density microarray, 630 novel ncRNA candidates were identified, 43 of which belong to the C/D and H/ACA-box subclasses of small nucleolar RNAs (snoRNAs) and small Cajal body–specific RNAs (scaRNAs) (Raabe 2010). Additionally, a family ncRNA was also found to be associated with the telomeres (Broadbent 2011). These transcripts are coordinately expressed after DNA replication in blood-stage parasites and may have a role in telomere maintenance and regulation of virulent gene expression (Bright 2011).

Advances and reduction in costs of high-throughput DNA sequencing have made it possible to sequence a large number of DNA and RNA samples (Bentley 2008; Rothberg 2008). RNA-sequencing analysis (RNA-seq) in *P. falciparum* generally refers to studies of genome-wide transcripts that use next-generation sequencing technologies (e.g., 454 sequencing or Illumina) to obtain a large amount of short cDNA sequences. The resulting sequence reads are either aligned to the reference genome or are *de novo* assembled to produce a genome-scale transcription map that consists of both transcriptional structure (sense and antisense strands) and level of expression for each gene in the genome. As the costs of genome sequencing continue to decrease, RNA-seq is likely to replace microarray as the choice of technique for genome-wide transcript analysis; particularly, transcripts from both parasites and their hosts can be obtained at the same time for analysis of parasite–host interaction using RNA-seq. In addition to studying gene expression pattern and functional prediction, these RNA-seq studies have also detected new transcripts, provided extensive data on alternative splicing, extended 3′ and 5′ untranslated regions, catalogued antisense or directional transcripts, and revealed genome-wide histone modifications throughout parasite intraerythrocytic development in laboratory and clinical isolates of *P. falciparum* (Bartfai 2010; Lopez-Barragan 2011; Otto 2010; Sorber 2010; Vignali 2011).

Epigenomics

Epigenomics is the study of genome-wide modifications of the genetic material of a cell, mostly DNA methylation and histone methylation and acetylation. Generally reversible without altering the DNA sequence, these modifications are involved in numerous cellular processes such as parasite differentiation, parasite development, and antigenic variation of parasite surface proteins. These studies have been made possible only recently through the adaptation of genomic high-throughput assays such as genome-wide analysis of chromatin immunoprecipitated DNA coupled with tiling microarray (ChIP-on-chip), or next-generation sequencing (Chip-Seq) (Park 2009). While not discussing the complex mechanisms of epigenetic regulation of gene expression, we would like to point out that although this field is still young and evolving for malaria parasites, significant progress has already been made. Readers interested in this subject can also see Chapter 14 or consult some recent excellent reviews on these subjects (Cui 2010; Duffy 2012; Dzikowski 2008; Hakimi 2007; Hoeijmakers 2012; Lopez-Rubio 2007; Merrick 2010). Similarly, techniques of proteomics and metabolomics can be applied to investigate parasite gene expression and gene function. These subjects are covered in chapters 8 and 9 and are discussed here.

Reverse genetics and parasite transformation

Genetic disruption or complementation of a gene of interest is very useful for studying gene function, particularly for malaria parasites that do not appear to have RNAi machinery (Baum 2009). Since development of methods for transformation of both *P. falciparum* (Wu 1995, 1996) and *P. berghei* (van Dijk 1996, 1995), parasite transformation and gene knockout have become routine processes in many laboratories. Again, we will not discuss individual studies but rather mention that there is an effort to collect genome-wide gene knockout using transposon-mediated mutagenesis (Balu 2006). For advances and modification on parasite transformation methods, readers can consult some reviews on the subjects (Balu 2012, 2006, 2007; Limenitakis 2011).

Summary

In the 10 years since completion of the *P. falciparum* genome sequences, the landscape of malaria research has dramatically changed. In most cases, there is no need to clone or sequence a gene or to perform a Southern or Northern blot. Instead, we have witnessed an explosion of genome-wide analyses of gene transcription, translation, modification, expression regulation, and GWAS. The trend will continue; at the same time, characterization of gene function will become the priority of scientific activity. Genomics approaches have been applied to many aspects of malaria research, such as drug resistance and parasite development. Future studies will need to focus more on parasite–host interaction, including disease severity and virulence.

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CHAPTER 6

Gene regulation: New insights and possible intervention strategies

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At any point during the life cycle of malaria parasites, only a subset of the approximately 5700 genes is expressed in monocistronic units. In the well-studied asexual blood stages, rapid cyclic activation of genes occurs at the transcriptional level; however, relatively few transcription factors have been identified to date, and their specific role in gene activation awaits validation. The discovery of histone modifications in malaria parasites and stage-specific proteomic studies have revealed the presence of additional layers of control upstream and downstream of basal gene transcription. These include epigenetic elements and post-transcriptional repression. Specifically, clonally variant gene families encoding immune evasion and housekeeping genes show a unique epigenetic control mechanism that allows only one member or a small subgroup of genes of a family to be expressed at a given time. The localization of such gene families to the subtelomeric regions of chromosomes further results in high phenotypic diversity in a population of genetically identical parasites. Insights into the enzymatic machinery that controls histone modifications involved in gene activation and repression have identified novel targets for the development of antiparasitic intervention strategies that could potentially interfere with all life cycle stages.

Introduction

The sequencing of the *Plasmodium falciparum* genome in 2002 (Gardner 2002) revealed that the 23.3 Mb genome is partitioned among 14 chromosomes and is highly AT-rich: about 79.6% throughout the genome, with introns and intergenic regions being up to 93% AT-rich. The latest annotation of the genome identified about 5700 open reading frames (ORFs), and nearly 60% of these lack homologues in other organisms, in spite of being conserved in other *Plasmodium* species. Subsequent transcriptomic analyses of the asexual (Bozdech 2003; Le Roch 2003) and the sexual stages (Young 2005) showed a cascade of gene expression with functionally related genes being transcribed at the same stage of the asexual lifecycle and a subset of genes being specifically expressed in the sexual stages. This suggested a mechanism of coordinated gene regulation and just-in-time transcription (see also chapter 8 by Gupta, Bozdech, and Preiser). However, due to the AT-richness of the genome, it has been difficult to accurately define 5′-untranslated regions (UTRs) and transcription start sites for most *P. falciparum* genes and to identify canonical *cis*-acting elements in the upstream regions of functionally related genes.

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One explanation for coordinated gene regulation could be polycistronic transcription, as is observed for the kinetoplastids *Trypanosoma brucei* and *Leishmania major* (Haile and Papadopoulou 2007). However, transcriptomic analyses do not support such a phenomenon, except maybe for transcription from the genomes of the mitochondria and apicoplast (Bozdech 2003; Ji 1996; Le Roch 2003). That transcription in *P. falciparum* is predominantly monocistronic, similar to most eukaryotes, was first realized from the work of Lanzer and colleagues in 1992 (Lanzer 1992a). The authors used nuclear run-on assays to demonstrate that a 40-kb genomic locus proximal to the glycophorin binding protein gene on chromosome 10 contained two ORFs that were transcribed monocistronically, albeit with the same directionality. In the same year, the authors also defined the transcriptional unit of the knob-associated histidine-rich protein (KAHRP) gene and identified a *cis*-acting element 160 bp upstream of the transcription start site that controlled stage-specific gene expression, further supporting monocistronic transcription (Lanzer 1992b). Although there is one report of bicistronic transcription for MAEBL, a member of the ebl family of proteins, and its upstream ORF PF3D7_1147700 (Balu 2009), the vast majority of chromosomal genes in *P. falciparum* are most likely monocistronically transcribed.

It has become clear that, similar to other eukaryotic organisms, *P. falciparum* possesses various layers of gene regulation, which could affect the timing of transcription and translation. These include epigenetic regulation through chromatin and possibly DNA modifications, modulation of gene expression by specialized transcription factors, alternative splicing, and finally, post-transcriptional, translational, and post-translational regulation (Figure 6.1). We discuss these mechanisms and the possibility of targeting some of these for novel malaria therapeutics in the following sections.

Modes of gene regulation

Epigenetic mechanisms of gene regulation

Chromatin structure

The nucleosome is the basic unit of chromatin structure in all eukaryotes and comprises an octamer of core histones, around which are wrapped 147 bp of DNA. *P. falciparum* has four canonical core histones – H2A, H2B, H3, and H4 – and four histone variants: H2A.Z, H2Bv, H3.3, and CenH3 (Miao 2006). A homologue for the linker histone H1 has not been identified in *Plasmodium* or other apicomplexans so far (Sullivan 2006). Nucleosomes modulate eukaryotic gene regulation by affecting the accessibility of other proteins to DNA (Kornberg and Lorch 1999). Two studies reported that nucleosome occupancy in *P. falciparum* was low in intergenic regions (Ponts 2010, Westenberger 2009), similar to other eukaryotes. However, a third study that used techniques optimized for the high AT content of the *P. falciparum* genome found that nucleosomal occupancy was comparable between coding and intergenic regions (Bartfai 2010). The single active member of the *var* gene family showed the lowest nucleosomal occupancy of all *var* genes (Westenberger 2009). Intriguingly, *var* introns presented a specific profile of chromatin changes over the *P. falciparum* life cycle, which was different from *var* promoters (Ponts 2010).

Overall, for the majority of genes, changes in nucleosome enrichment did not correlate with gene expression (Ponts 2010; Westenberger 2009). Therefore, the cyclic cascade of steady-state mRNA levels observed during the parasite's intra-erythrocytic life cycle (Bozdech 2003; Le Roch 2003) cannot be explained by fine remodeling of chromatin structure. However, analysis of chromatin architecture at transcription start sites (TSSs) and predicted core promoters showed that on the whole, these regions are nucleosome-free in asexual stages, except for genes exclusively expressed during sexual

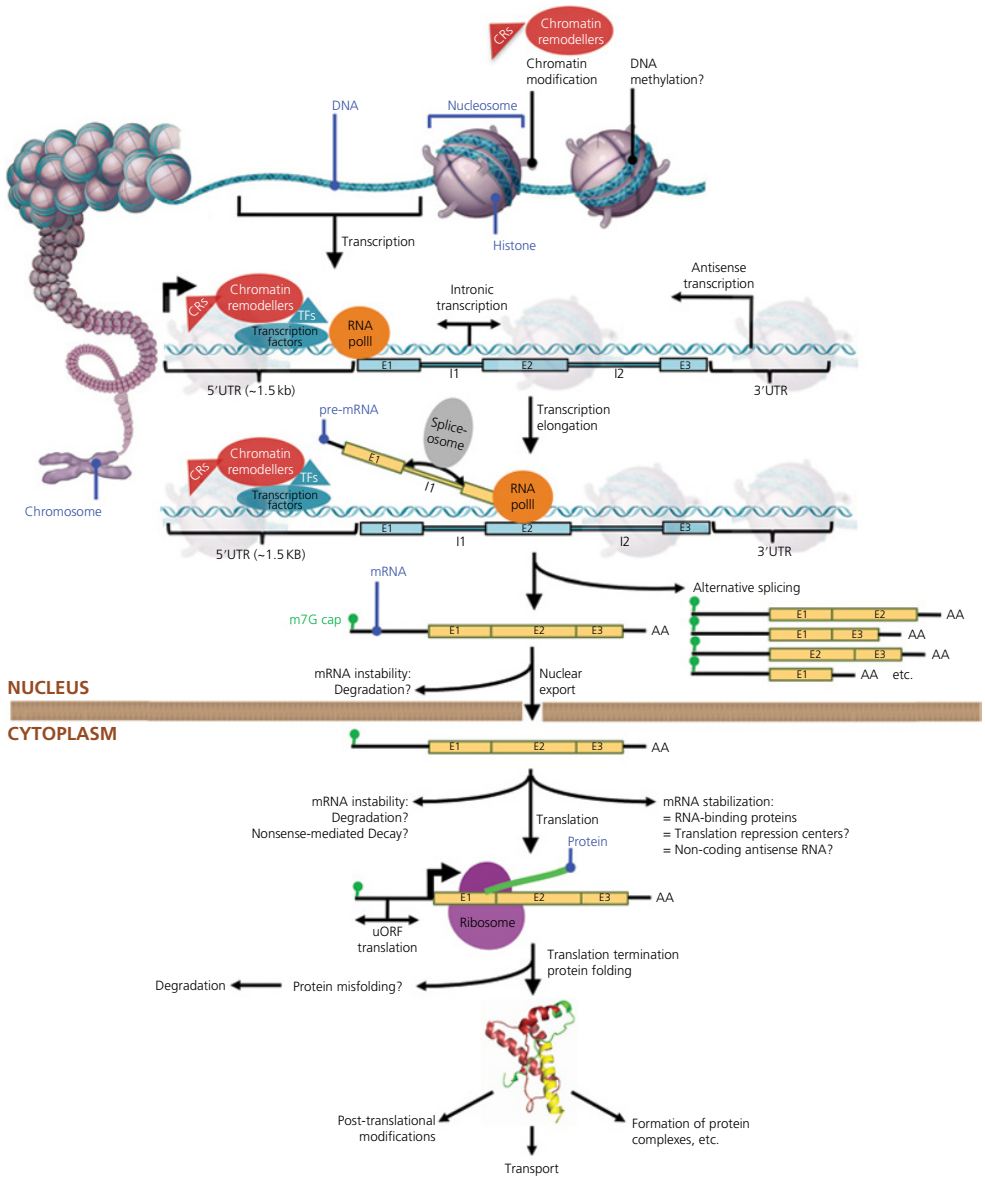

Figure 6.1 Multiple layers of gene regulation exist in *P. falciparum*.

stages, which contain a single nucleosome within these loci (Ponts 2011). A point of contention is nucleosomal occupancy dynamics in subtelomeric regions (Table 6.1): Ponts *et al.* reported an overenrichment of nucleosomes for telomeric and subtelomeric regions in comparison with the rest of the genome, and Westenberger *et al.* observed that subtelomeric regions showed the highest fluctuations in nucleosome occupancy, that is, low in early stages of the asexual life cycle and high in late trophozoites and schizonts.

Table 6.1 Functional chromatin compartmentalization.

Feature/Protein	Core chromosomes	Subtelomeric regions
Chromatin structure	Low levels of nucleosomes	High levels of nucleosomes
	Smaller fluctuations along the cycle	Larger fluctuations along the cycle
	No correlation between gene expression and nucleosome enrichment	Inverse correlation between transcriptional activity and nucleosome occupancy
Heterochromatin mark H3K9me3	Depleted of this mark except for a few heterochromatin islands	Specifically enriched
Euchromatin marks	Highly enriched	Depleted except for a few active loci
PfHP1	Depleted, except at the heterochromatin islands	Specifically enriched
PfSir2	Depleted	Enriched
PfH2A.Z	Highly enriched at intergenic regions	Depleted at intergenic regions except at the transcriptionally active *var* promoter
Nuclear organization	Mainly central except for heterochromatin islands	Perinuclear

Histone modifications

In contrast to the paucity of specialized transcription factors (see section below), *P. falciparum* has a rich complement of chromatin-modifying proteins, suggesting that histone modifications might play an important role in the control of gene expression. In fact, mass spectrometric analyses have shown that plasmodial histones carry more than 60 post-translational modifications, including acetylation, methylation, and phosphorylation (Dastidar 2012; Treeck 2011; Trelle 2009); however, only a few have been studied in depth. Much of our knowledge about the role of chromatin modifications in transcription regulation in *P. falciparum* comes from the investigation of the mutually exclusive expression of the *var* gene family (for a recent review, see Guizetti and Scherf 2012).

Briefly, the *var* gene family is composed of 60 members that encode for the major virulence adhesion surface molecule PfEMP1. Only one *var* gene is expressed at any given time, and switching expression among the 60 members avoids immune clearance and prolongs the period of infection and subsequent transmission to the mosquito. The transcriptionally active *var* locus associates with acetylation of lysine 9 on histone H3 (H3K9Ac) and di- and trimethylation of lysine 4 on the same histone (H3K4me2 and H3K4me3, respectively) (Figure 6.2) (Lopez-Rubio 2007). In contrast, silenced *var* genes are highly enriched in the heterochromatin mark trimethylation of lysine 9 on histone H3 (H3K9me3) (Chookajorn 2007; Lopez-Rubio 2007; Lopez-Rubio 2009). An active *var* locus is clonally inherited over many life cycles: During each cycle, the active *var* gene is transcribed between 4 and 20 hours after erythrocytic invasion, but in later stages it is transiently repressed but remains in a state poised for transcription in the next cycle, maintaining the enrichment in H3K4me2 (Lopez-Rubio 2007). Therefore, the interplay of active and repressive histone marks serves as a long-term signature for the propagation of the active and silenced state, respectively, of *var* genes through cell division.

Clonally variant gene expression in *P. falciparum* occurs beyond *var* genes and predominantly affects gene families located in subtelomeric regions (Scherf 2008). These not only include large gene families such as *rif, stevor,* and *pfmc-2tm* encoding erythrocyte membrane-associated proteins, but also include smaller gene families encoding proteins involved in diverse cellular functions such

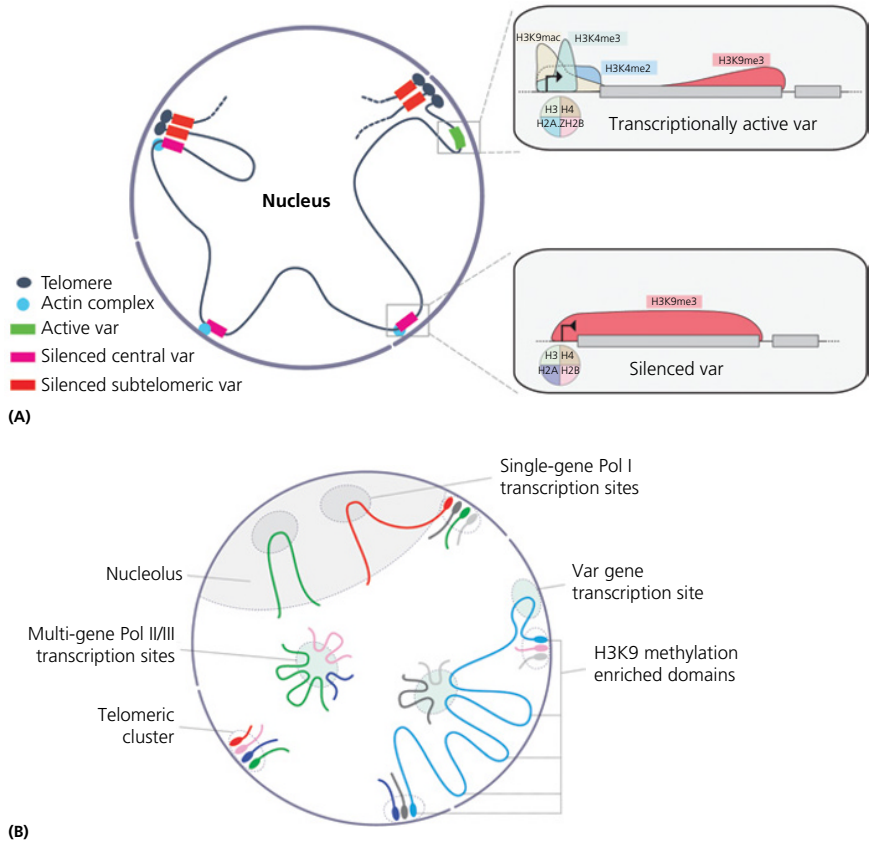

Figure 6.2 A) Schematic representation of histone marks and histone variants linked with silent and active *var* gene loci. A similar situation applies to other clonally variant gene families. The spatial organization of the *var* gene family in the nucleus is also illustrated. B) Model for the nuclear organization of *P. falciparum* genomic DNA at the ring stage indicating the major nuclear compartments: nucleolus, Pol I and Pol II/III transcription sites, telomeric clusters, and chromosome organization. (Adapted from Mancio-Silva 2010.) (*See insert for color representation of this figure*.)

as erythrocyte invasion (*eba*, *PfRh*, and c*lag*) (Cortes 2008). Genome-wide mapping showed that spatially, the H3K9me3 mark is specifically associated with silent clonally variant gene families (Lopez-Rubio 2009; Salcedo-Amaya 2009). This is in contrast to higher eukaryotes, where H3K9me3 is a general epigenetic mark found in many constitutive heterochromatin regions of the genome (Grewal and Jia 2007). The activation marks H3K4me3 and H3K9Ac, on the other hand, showed a broad distribution, marking 91% of the *P. falciparum* genome (Lopez-Rubio 2009; Salcedo-Amaya 2009). This correlated with other unicellular eukaryotes that have a predominantly euchromatic epigenome and diverged from mammals that have about 60% of their genome permanently silenced (Garcia 2007). Furthermore, analysis of the temporal profile of these marks showed that H3K9Ac dynamics at promoter regions follows the pattern and level of steady-state mRNA expression throughout intraerythrocytic development, whereas H3K4me3 does not. Instead, H3K4me3 appears to be deposited in a stage-specific manner, that is, low at early stages and high at mature stages (Bartfai 2010) and rather marks genes that are actived at one stage during the asexual cycle, similar to the poised *var* gene (Lopez-Rubio 2007).

Histone variants

Histone variants assemble into nucleosomes to create functionally specialized chromatin domains (Talbert and Henikoff 2010). The histone H2A variant H2A.Z binds to diverse genomic sites and has apparently opposing roles, some of which could be explained by post-translational modifications of H2A.Z nucleosomes (Talbert and Henikoff 2010). In *P. falciparum*, PfH2A.Z is present at intergenic regions of the euchromatic genome along with H3K4me3 and H3K9Ac, and it may be associated with the general strength of promoters but not their temporal activity (Bartfai 2010). In addition, PfH2A.Z has a distinct role in *var* gene activation: In early intra-erythrocytic stages, the promoter region of the transcriptionally active *var* shows high levels of PfH2A.Z (Figure 6.2). This association is lost during later stages, when the active *var* is in the poised state (Petter 2011). Given that PfH2A.Z contained nine different post-translational modifications, these may be important to regulate its function (Dastidar 2012; Trelle 2009). Genome-wide mapping of the centromeric histone variant PfCenH3 showed that it occupies the centromere of each chromosome. This region is also enriched in PfH2A.Z but is devoid of pericentric heterochromatin marks such as H3K9me3 and H4K20me3 (Hoeijmakers 2012; Lopez-Rubio 2009). PfH3.3 and PfH2Bv have not been studied in *P. falciparum* but present several euchromatic modifications (Dastidar 2012; Treeck 2011; Trelle 2009).

Trans-factors associated with histone modifications and chromatin structure

The *P. falciparum* genome is predicted to encode a large number of histone-modifying proteins or epigenetic writers (Aravind 2003; Gardner 2002); however, only a few of these have been functionally characterized. Of the eight putative histone acetyltransferases (HATs) that have been identified bioinformatically (Bischoff and Vaquero 2010), PfGCN5 and PfMYST are the best studied. PfGCN5, which preferentially acetylates histone H3 at K9 and K14 (Fan 2004) has a genome-wide distribution similar to that of H3K9Ac (Cui 2007b). PfMYST, on the other hand, exhibits a preference to acetylate histone H4 at K5, K8, K12, and K16. Both proteins appear to be essential for asexual intraerythrocytic growth, with PfGCN5 inhibition leading to arrest of parasite development (Cui 2007a) and PfMYST overexpression leading to disruption of cell cycle regulation and DNA repair (Miao 2010a). Interestingly, PfMYST was recruited to the transcriptionally active *var* promoter (Miao 2010a), correlating with the observation that upstream regions of the active *var* gene associate with acetylation of histone H4 (Freitas-Junior 2005).

Histones are deacetylated by histone deacetylases (HDACs). In *P. falciparum,* there are five HDACs, including two paralogues PfSir2A and PfSir2B of Sir2, the class III NAD^+-dependent HDAC sirtuins. PfSir2A and PfSir2B work in concert with acetylated histones to silence different groups of *var* genes (Duraisingh 2005; Freitas-Junior 2005; Tonkin 2009a). Substrates of PfSir2A include acetylated K9 and K14 of histone H3 and acetylated K16 of histone H4 (French 2008). Furthermore, along with the origin-of-recognition-complex 1 molecule of *P. falciparum* PfOrc1, PfSir2A is involved in the propagation of silent heterochromatin up to 20 to 30 kb from the telomere, causing silencing of proximal genes such as *var* by the telomere position effect (Duraisingh 2005; Freitas-Junior 2005; Mancio-Silva 2008). A novel role has been reported for PfSir2A in modulating ribosomal RNA (rRNA) transcription (Mancio-Silva 2013). Because PfSir2A localizes to the rRNA gene-containing nucleolus in addition to heterochromatin, and because it is a NAD^+-dependent sirtuin, host diet-induced changes in the parasite´s NAD^+/NADH ratio may affect parasite multiplication by limiting PfSir2A-mediated rRNA expression. In turn, high temperature and lactate levels in patients correlate with elevated PfSir2A expression, and parasites overexpressing PfSir2A show deregulated expression of a subset of *var* genes during *in vitro* culture (Merrick 2012). Taken together, this suggests that altering the activity or/and expression of PfSir2A could modify the parasite's capacity for proliferation and virulence, making PfSir2A a potential therapeutic target.

Methylation of histones is catalyzed by histone arginine or lysine methyltransferases: PRMTs and HKMTs, respectively. Of the three conserved PRMTs in *P. falciparum*, only PfPRMT1 has been characterized. PfPRMT1 localizes to the nucleus and cytoplasm of intra-erythrocytic parasites and targets arginine 3 of histone H4 for methylation, although it also demonstrated methyltransferase activity toward other non-histone protein substrates (Fan 2009b). The HKMTs, in turn, can be divided into two major families: SET domain–containing enzymes and those related to Dot1. In *P. falciparum*, while no *Dot1* orthologue is evident, ten SET domain–containing HKMTs have been found (Bischoff and Vaquero 2010; Cui 2008a). Biochemical and localization studies have tried to provide insight into their potential functions (Cui 2008a; Volz 2010). Specifically, the putative H3K9me3 methyltransferase PfSET3/PfKMT1 localizes at the nuclear periphery and appears to be essential for parasite proliferation (Lopez-Rubio 2009; Lopez-Rubio and Scherf, unpublished data). In contrast, PfSET10, which methylates H3K4, colocalizes with the active *var* in post-ring stages, indicating a role for PfSET10 in maintaining the active *var* in a poised state during mature stages (Volz 2012).

Opposing the action of histone methyltransferases are histone demethylases. *P. falciparum* has three orthologues of the two categories of these enzymes: lysine-specific demethylases (LSD1) and the Jumonji-C histone demethylases (JHDM) (Cui 2008a; Volz 2010). The functions of these enzymes have not been investigated, although nuclear localization has been reported for PfLSD1 (Volz 2010).

The aforementioned epigenetic writers are complemented by a wide variety of epigenetic readers, proteins that contain functional domains that bind to modified (or unmodified) amino acid side chains. These include bromodomains, which bind to acetylated lysine residues, chromodomains, and PHD domains, which bind to methylated lysine residues, and 14-3-3 proteins, which bind to phosphorylated serines and threonines. *P. falciparum* has more than 15 proteins containing chromatin binding domains (Bischoff and Vaquero 2010; Volz 2010). A well-studied chromodomain-containing protein in *P. falciparum* is the structural protein heterchromatin protein 1 (PfHP1). PfHP1 binds to H3K9me3 *in vitro* and its enrichment correlates with H3K9me3 throughout the genome, indicating that PfHP1 silences *var* and other gene families by binding to H3K9me3 (Flueck 2009; Perez-Toledo 2009). An added level of complexity is brought about by the fact that many histone-modifying writer enzymes also contain reader domains (Kouzarides 2007). For example, the HAT PfMYST harbors a chromodomain and the HKMT PfSET10 harbors a PHD domain.

Histone phosphorylation is known to be an important epigenetic mark associated with several cellular eukaryotic processes (chromosome condensation, DNA replication, and transcriptional regulation, among others), and its presence has been shown in plasmodial histones (Dastidar 2012b; Treeck 2011). Additionally, studies have shown that the *P. falciparum* protein kinase CK2 phosphorylates histones extracted from parasites *in vitro* (Dastidar 2012a), and of the three proteins containing 14-3-3 phosphoserine-binding domains, only Pf14-3-3I selectively binds to histone H3 peptides phosphorylated at serine 28 *in vitro* (Dastidar 2012b). Further studies need to be performed to explicate the role of histone phosphorylation in this parasite.

The *P. falciparum* genome encodes for several chromatin-remodeling molecules. These proteins regulate the spatial and temporal removal and movement of nucleosomes and deposition of histone variants, thus affecting the transcriptional status of the remodeled locus (Bischoff and Vaquero 2010). Although our knowledge of their functionality in *P. falciparum* is limited, the localization and initial characterization of some chromatin remodellers (or their orthologues in other apicomplexa) have been reported (Duffy 2012; Horrocks 2009a).

A novel DNA/RNA-binding protein family (four members in *P. falciparum*) that bears homology to the archaeal protein Alba (*a*cetylation *l*owers *b*inding *a*ffinity) was recently identified (Chene 2012a). PfAlba proteins were enriched at the nuclear periphery and partially colocalized with PfSir2.

The nuclear location changed at the onset of parasite proliferation (trophozoite–schizont), where the PfAlba proteins were also detectable in the cytoplasm in a punctate pattern. Discovery of the PfAlbas may provide a link between the previously described subtelomeric noncoding RNA and the regulation of antigenic variation (Chene 2012a; Goyal 2012).

Nuclear organization

The eukaryotic nucleus is a spatially organized compartment, with gene localization regulating expression (Gasser 2001). Similarly, in *P. falciparum*, nuclear organization dynamics appear to be important in gene regulation, especially for clonally variant genes families (see the model in Figure 6.2).

The most prominent intranuclear domain is the nucleolus, which is formed upon transcription of the ribosomal DNA (rDNA) into rRNA by RNA polymerase I. In *P. falciparum*, although single rDNA units are spread out on different chromosomes, they cluster in a single perinuclear nucleolus in pre-replicative parasite blood stages, regardless of their transcriptional status (Mancio-Silva 2010). Because this clustering is restricted to chromosome ends bearing rDNA units, it creates a physical constraint for chromosomal interactions and shapes nuclear organization of the parasite genome (Figure 6.2A) (Mancio-Silva 2010).

A second intranuclear domain in *P. falciparum* is composed of sites of RNA polymerase II- and III-mediated transcription, as visualized by bromouridine (BrdU) incorporation into nascent RNA in permeabilized live parasites (Mancio-Silva 2010). In early asexual stages, there are few active transcription foci in internal and peripheral nuclear regions; this number increases significantly during later stages of the life cycle (see the schematic in Figure 6.2B). The use of a specific inhibitor for RNA polymerase II and III reduced the number of transcription foci in early stages to two perinuclear foci that associate with the nucleolus and correspond to the active rDNA genes, further evidencing the presence of this second domain (Mancio-Silva 2010). Overall, it is speculated that the foci comprising this second intranuclear domain accommodate the transcription of co-regulated genes regardless of their chromosome location due to shared regulatory motifs and proteins (Iengar and Joshi 2009; Mancio-Silva 2010).

Mutually exclusive expression of *var* genes is also regulated by their subnuclear organization. As in *S. cerevisiae*, *P. falciparum* telomeres form physical clusters that are anchored to the nuclear periphery (Freitas-Junior 2000). Subtelomeric, silenced *var* genes colocalize with telomeres to the nuclear periphery, forming four to seven foci of chromosome ends, and internal *var* genes locate to the perinuclear space independent of telomeric clustering (Lopez-Rubio 2009). Two studies have provided important insights into the mechanism of perinuclear anchoring of these regions: In the first study, the repressive histone H3K9me3 mark and the responsible enzyme (PfSET3/KMT1) were enriched in telomeric clusters (Lopez-Rubio 2009) and were thought to determine anchoring. This is in concert with the observation that SpKMT1 is essential for the correct subnuclear localization of the silent mating type locus in *Schizosaccharomyces pombe* (Alfredsson-Timmins 2007). In the second study, the recruitment of central *var* genes to the nuclear periphery involves indirect binding of perinuclear actin-I to the *var* intron, probably via the ApiAP2 transcription factor PF3D7_1107800 (Zhang 2011), demonstrating a critical role for actin polymerization in the repositioning of *var* loci and *var* gene derepression. In contrast, the activation of a *var* gene requires its relocation to a transcriptionally competent area, still at the nuclear periphery but different from the silent cluster (Figure 6.2A) (Lopez-Rubio 2009; Ralph 2005; Voss 2006). Observations from rare cases where two *var* promoters are active showed that both loci share the same compartment at the nuclear periphery (Brolin 2009; Dzikowski 2007; Zhang and Scherf unpublished data). Taken together, these results hint at the existence of a unique perinuclear domain for transcription of the active *var* gene. This compartement may also accommodate active members of other clonally variant gene families such us *rifin* (Howitt 2009).

In higher eukaryotes, the nuclear pore plays a key role in activating gene transcription. High-resolution 3D analysis of nuclei during *P. falciparum* intraerythrocytic growth showed coupled dynamics of asexual development and nuclear pore organization (Weiner 2011). It is tempting to speculate that nuclear pore complexes in *P. falciparum* might regulate gene activation.

DNA methylation

In eukaryotic cells from different kingdoms, DNA methylation at the C5 position of cytosine (m5C), mostly within CpG dinucleotides, is a stably inherited epigenetic mark and is associated with a variety of functions including transcriptional regulation and control of genomic stability (Jones 2012). The extent of DNA methylation differs between different species: For example, 4% of all cytosines are methylated in human fibroblast cells, whereas in *Drosophila melanogaster*, it is just 0.05% to 0.1% (Gowher 2000; Lister 2009). In *P. falciparum,* the existence of DNA methylation remains unclear. Low cytosine methylation was first reported in 1991 using methylase-sensitive restriction analyses (Pollack 1991), and more recently, much higher levels of DNA methylation was reported using bisulfite sequencing (Ponts 2010b). Nonetheless, mass spectrometric analyses have failed to identify methylated nucleotides in *P. falciparum* (Choi 2006). Given that a single DNA methyltransferase (Dnmt) orthologue that belongs to the Dnmt2 protein family has been found in the *P. falciparum* genome (Templeton 2004), further studies are needed to determine whether DNA methylation indeed exists in *P. falciparum*. Interestingly, human Dnmt2 appears to be a tRNA methyltransferase rather than a Dnmt (Goll 2006), and the *Entamoeba hystolitica* and *D. melanogaster* Dnmt2 homologues have been proposed to have dual specificity for DNA and tRNA (Fisher 2004; Jeltsch 2006). Therefore, PfDnmt2 might be specific for tRNA and not DNA, and this remains to be elucidated.

Transcription factors and other DNA-binding proteins

P. falciparum promoters resemble canonical eukaryotic promoters: They contain a binding site for the preinitiation complex proximal to the transcription start site (TSS) and a second regulatory upstream region that either enhances or represses transcription (Horrocks 1998). Whereas this structure seems to be conserved across *P. falciparum* genes, the distances of the TSS to the initial ATG and the regulatory element to the TSS are variable (Horrocks 2009b). Moreover, only a few known regulatory DNA motifs have been experimentally identified: an SV40 element upstream of the glycophorin binding protein 30 (gbp30, (Lanzer 1992a)) and three conserved motifs in *var* gene promoters, namely the subtelomeric *var* gene promoter elements SPE1 and SPE2 and the central *var* gene promoter element CPE (Voss 2003). Evidence for protein complexes binding to them is available from electromobility shift assays (EMSAs), but the identity of these *trans*-acting factors remains elusive.

With the availability of the *P. falciparum* genome sequence, bioinformatics searches identified a number of motifs for binding sites of putative transcription-associated factors (TAF) (Gunasekera 2007; Wu 2008; Young 2008). Moreover, a comparison of promoters in functional gene clusters of different murine *Plasmodium* species and *P. falciparum* identified 81 conserved motifs, suggesting that phylogenetically conserved TAFs might regulate their expression (Imamura 2007).

Transcription factors in *P. falciparum*

The first attempt to define TAFs in *P. falciparum* by mining genomic sequences using hidden Markov model (HMM) profiles for transcriptional regulators (Coulson 2004) found 69 matches, representing 1.3% of the genome. This was much lower than in other eukaryotes: For example, in *Saccaromyces cerevisiae* and *Homo sapiens*, 4% and 6.8% of all proteins, respectively, encode for TAFs. Then, in 2005, the ApiAP2 family of specific transcription factors was discovered in apicomplexan parasites,

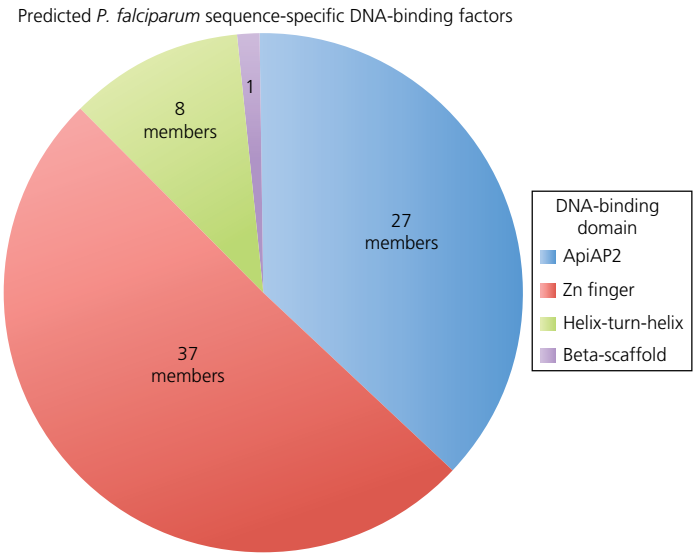

Figure 6.3 A pie chart summary of putative transcription factors in the genome of *P. falciparum* as determined by bioinformatic analyses.

expanding the number of predicted TAFs (Balaji 2005). A scan of the *P. falciparum* genome for TAFs used additional HMM profiles, including those from Coulson (2004), the hydrophobic cluster analysis from Callebaut (2005), and the ApiAP2 proteins from Balaji (2005), and retrieved a total of 202 proteins comprising 3.5% of the genome (Bischoff and Vaquero, 2010). These included proteins associated with the general transcriptional machinery, 73 specific TAFs and chromatin-associated proteins. The specific TAFs further comprised 27 AP2-domain-containing proteins, 37 proteins belonging to the CCCH-type zinc-finger family, 8 helix-turn-helix proteins and 1 beta-scaffold factor with minor groove contacts (Figure 6.3). The DNA-binding proteins for which experimental validation has been obtained are summarized in Table 6.2 and are described below.

PfMyb1

PfMyb1, the first specific TAF described in *P. falciparum* (Boschet 2004), belongs to the tryptophan-cluster family of helix-turn-helix transcription factors (Bischoff and Vaquero 2010). When the *P. falciparum* genome was BLASTed with the consensus sequence from an alignment of the DNA-binding domains of more than 200 non-redundant eukaryotic Myb proteins, PfMyb1 was discovered (Boschet 2004). Similarly, the putative Myb regulatory element (MRE) was identified in several *P. falciparum* promoters by alignment with the DNA-binding site of chicken Myb and was shown to bind to *P. falciparum* nuclear proteins by EMSAs (Boschet 2004). Using an antisense approach, reduction of PfMyb1 levels in asexual parasites was shown to impair growth, specifically, trophozoite to schizont transition (Gissot 2005). Loss of PfMyb1 also resulted in the differential expression of 11 genes including kinases, one phosphatase, a TATA-binding protein homologue, and histones (Gissot 2005). The binding of PfMyb1 to the promoters of some of those genes was confirmed by chromatin immunoprecipitation (ChIP), providing the first characterization of a specific TAF in *Plasmodia*.

The APiAP2 proteins

Balaji *et al*. mined several apicomplexan genomes using position-specific weight matrices and HMMs and identified a conserved domain containing one DNA-binding motif, the AT-hook, that presented significant homology to the plant Apetala 2 (AP2) DNA-binding domain, a domain also found in endonucleases and integrases (Balaji 2005). Thus was discovered the ApiAP2 family, with

Table 6.2 Validated *Plasmodium* DNA-binding proteins regulating transcription and/or chromatin organization.

DNA-binding protein	PlasmoDB accession number	*Plasmodium* species	TRANSFAC* classes/ subclasses	Biological processes	Reference
PfMyb1	PF3D7_1315800	*P. falciparum*	Specific transcription factor/HTH	Transcriptional regulation of genes involved in cell cycle regulation/progression	Gissot 2005
AP2-O	PBANKA_090590	*P. berghei*	Specific transcription factor/ApiAP2	Transcriptional regulation of genes involved in ookinete development	Yuda 2008
AP2-Sp	PBANKA_132980 PF3D7_1466400	*P. berghei* *P. falciparum*	Specific Transcription factor/ApiAP2	Transcriptional regulation of genes involved in sporozoite development	Campbell 2010 Yuda 2010
AP2-L	PBANKA_021440	*P. berghei*	Specific transcription factor/ApiAP2	Transcriptional regulation of liver-stage genes	Iwanaga 2012
PfSIP2	PF3D7_0604100	*P. falciparum*	Specific transcription factor/ApiAP2	Binds subtelomeric noncoding regions	Flueck 2010
PfAP2-var^{int}	PF3D7_1107800	*P. falciparum*	Specific transcription factor/ApiAP2	Binds to an intron element of *var* genes in complex with nuclear actin	Zhang 2011
PfAlbas	PF3D7_0814200 PF3D7_1346300 PF3D7_1006200 PF3D7_1347500	*P. falciparum*	Alba	Non-specific DNA binding but enriched in subtelomeres	Chene 2012 Goyal 2012
Pf/PyHMGBs	PF3D7_1202900 PF3D7_0817900 PY07077	*P. falciparum* *P. yoelii*	Chromatin-remodeling factor/HMGB	Non-specific DNA binding; regulates transcription of gametocyte genes and ookinete formation	Briquet 2006 Kumar 2008 Gissot 2008

*TRANSFAC: transcription factors database: http://www.generegulation.com/pub/databases.html

P. falciparum presenting 27 members containing anywhere from one to three AP2 domains (Campbell 2010). Interestingly, when the transcriptional profiles of the *P. falciparum* proteins were compared, they were found to predominantly map to four stage-specific clusters during the intraerythrocytic life cycle, indicating that the PfApiAP2s might be key transcriptional regulators of stage-specific genes (Balaji 2005).

Comparison of the secondary structure of the ApiAP2 domains showed conservation of the three consecutive beta strands and alpha helix that comprise the GCC-box binding domain of an AP2 protein from *Arabidopsis thaliana* (Balaji 2005; De Silva 2008). Importantly, in spite of the core fold of the AP2 domain being conserved among the ApiAP2 proteins, the residues that contact DNA are not conserved, suggesting that the motifs recognized by these proteins might be distinct.

In accordance with this, in a recent study, individual PfApiAP2 domains were expressed as recombinant proteins and their binding specificity analyzed using DNA oligonucleotide arrays (Campbell 2010); distinct AP2 domains, including those from the same *P. falciparum* protein, were found to bind to different motifs. Taken together with the observation that the PfApiAP2s themselves are expressed in a stage-specific manner (Balaji 2005), sequence specificity could allow a small number of proteins to regulate transcription throughout the life cycle of the parasite.

The first report demonstrating the DNA-binding activity of two orthologous ApiAP2 proteins containing one and two AP2 domains from *P. falciparum* and the distantly related apicomplexan *Cryptosporidium parvum*, respectively, demonstrated that the orthologues of the two proteins bound to identical sequences and that binding was independent of the presence of one or two AP2 domains, contrary to what is observed in plants (De Silva 2008). Since then, several other PfApiAP2 proteins have been investigated. Of particular interest are PfSIP2, PF3D7_1143100, and PF3D7_1469500, the recognition sequences for which are enriched in the *var* promoter (Campbell 2010). The well-characterized PfApiAP2 protein PfSIP2 was identified in a protein complex that bound to the SPE2 elements present in subtelomeric *var* genes (Flueck 2010). The full-length protein contains 1979 amino acids and two tandem AP2 domains, and is expressed and proteolytically processed in late intraerythrocytic stages. By ChIP analysis, PfSIP2 was found to occupy only a fraction of the predicted binding sites in the parasite genome, mostly in the subtelomeric region. Because the disruption of the gene was not successful, the authors over-expressed PfSIP2's AP2 domains and found no effect on transcriptional regulation; conversely, the protein was found to colocalize with PfHP1, both by immunofluorescence microscopy and ChIP. This was the first observation of a putative transcription factor being linked to heterochromatin in *P. falciparum*.

Although PF3D7_1143100 and PF3D7_1469500 are not well studied, their *P. berghei* orthologues, PbAP2-O (expressed in ookinetes) and PbAP2-Sp (expressed in sporozoites), respectively, function as true TAFs (Yuda 2010; Yuda 2009). A parasite line deficient for PbAP2-O showed downregulation of zygote and ookinete genes, and binding of PbAP2-O to the promoters of these genes was confirmed by ChIP (Yuda 2009). In contrast, deletion of PbAP2-Sp resulted in parasite lines unable to produce sporozoites, and ChIP assays could not be performed due to limiting amounts of sporozoite nuclear extracts (Yuda 2010). To circumvent this limitation, the authors swapped the AP2 domains of PbAP2-O and PbAP2-Sp, and they observed the induction of sporozoite genes in the ookinete stage, indicating that the AP2 domain of PbAP2-Sp is indeed required to activate sporozoite-specific genes. Moreover, the chimeric PbAP2 bound to promoters of sporozoite-specific genes by ChIP when ookinete extracts of parasites expressing the chimeric protein were used.

Another AP2 member, termed AP2-L, has been characterized in *P. berghei* (Iwanaga 2012). Although the protein was expressed in blood-stage trophozoites, salivary-gland sporozoites, and post-hepatocyte invasion, AP2-L knockout parasites began showing developmental defects in liver stages only 36 hours after invasion. In line with this phenotype, transcriptional changes in the knockout line were also only observed in liver stages.

PF3D7_1107800 was identified by mass spectrometry in a protein complex that binds to a *var* intron element that tethers the *var* introns to the nuclear periphery (Zhang 2011). Although this complex also contains actin-I, recombinant actin was not able to shift the *var* intron element in EMSAs. Instead, the AP2 domain of PF3D7_1107800 was able to bind with sequence specificity to the intron element (Zhang 2011), and data from ChIP analyses of early asexual stage parasites indicate that the protein indeed binds to *var* introns *in vivo* (Martins RM and Scherf A, unpublished data).

PfAlbas: Nonspecific DNA/RNA-binding proteins

A novel DNA/RNA-binding protein family that bears homology to the archaeal protein Alba has been identified in *P. falciparum* (Chene 2012a). The four PfAlbas bind DNA and RNA with relaxed sequence specificity and localize to the nuclear periphery in early asexual stages (Chene 2012).

Moreover, ChIP analyses showed that PfAlba3 localizes to telomeric and subtelomeric regions as well as to the *var* promoter *in vivo* and inhibits transcription *in vitro* (Goyal 2012). Given that Alba proteins have the ability to homo- and heterodimerize and that PfAlba1, PfAlba2, and PfAlba4 each have a second distinct subdomain, each PfAlba may have evolved a specific task in chromatin and RNA biology of *P. falciparum*.

High-mobility-box group (HMBG) proteins

Classically, HMGBs bind to noncanonical DNA structures with no apparent sequence specificity and induce conformational changes, in turn regulating transcription (Stros 2010; Ueda and Yoshida 2010). The *P. falciparum* genome has four annotated HMGB proteins, but only two of them have characterized DNA-binding activities: PfHMGB1 and PfHMGB2 preferentially bind to quadruplex DNA molecules (Briquet 2006) and to cisplatin-crosslinked DNA (Kumar 2008), the former being expressed in asexual forms of the parasite and the latter being expressed in gametocytes. Currently, no evidence exists for a direct role of PfHMGBs in gene regulation. However, there is evidence that HMGB2 from the murine parasite *P. yoelli* modulates transcription of gametocyte-specific genes (Gissot 2008).

Alternative splicing

One of the first decisions made during the synthesis of a pre-mRNA molecule in eukaryotes is alternative splicing (Figure 6.1); this results not only in an expansion of the protein repertoire of an individual cell, but it also regulates protein isoforms in a cell-, tissue-, and organ-specific manner in higher eukaryotes or in response to external stimuli. Given that nearly 50% of *P. falciparum* genes have introns, with over half of these possessing at least two introns (PlasmoDB), alternative splicing is predicted to be an important modulator of gene expression in *P. falciparum*. The first publication of alternative splicing in *P. falciparum* blood stages reported three isoforms of the 41-3 antigen-coding precursor RNA, which contains eight introns (Knapp 1991). Since then, several groups have demonstrated that alternative splicing does occur during the asexual and sexual stages of the parasite life cycle for genes such as adenylyl cyclase (Muhia 2003), maebl (Singh 2004), the apicoplast-targeted stromal-processing peptidase and delta-aminolevulinic dehydratase (van Dooren 2002), and so on, and affects properties as varied as protein function, solubility, and localization.

A targeted small-scale study of alternative splicing (Iriko 2009) and RNA-seq analysis (Otto 2010) of asexual stages of *P. falciparum* initially described a total of 84 such events. Subsequently, a systematic RNA-seq-based genome-wide study of splicing in intra-erythrocytic stages detected about 310 alternative splicing events in 254 (4.5%) *P. falciparum* genes (Sorber 2011). The overall outcome of these events was predicted to be the synthesis of truncated proteins (the predominant outcome), multiple protein isoforms (up to 16 for some genes), and downstream post-transcriptional regulation. Interestingly, the authors also found that although some introns were never spliced, a subset of antisense transcripts was alternatively spliced; they suggest that this could result either in as-yet-unannotated genes or in the discovery of novel gene regulatory mechanisms.

To achieve alternative splicing, eukaryotes use different splicing regulators, most notably the serine/arginine-rich (SR) proteins (Chen and Manley 2009). The SR proteins are in turn regulated by phosphorylation by the SR protein-specific kinases (SRPKs) and cyclin-dependent kinase–like kinases (CLKs). Studies have identified the SRPK homologue PfSRPK1 and its substrate PfSR1 as splicing factors in *P. falciparum* (Dixit 2011; Eshar 2012). The researchers demonstrated that the phosphorylation of PfSR1 by PfSRK1 reduces its RNA-binding capacity *in vitro*, that PfSR1 is a *bona fide* splicing enhancer in mRNA splicing assays, and that the localization of PfSR1 is developmentally regulated during the intra-erythrocytic lifecycle of *P. falciparum*. PfSR1's critical role was further evident from the misregulation of alternative splicing that occurred upon PfSR1 overexpression, resulting in a disruption of intra-erythrocytic development

(Eshar 2012). Taken together with the observation that PfASF-1, another SR protein, is a putative substrate for PfCLK1 and PfCLK2 (Agarwal 2011) and that there are more than 10 SR and SR-like proteins encoded in the *P. falciparum* genome (Sorber 2011), it will be important to explicate the role of alternative splicing and splicing regulators in fine-tuning gene expression and regulating parasitic growth.

Post-transcriptional, translational, and post-translational gene regulation

Once a pre-mRNA molecule has been processed to its capped, spliced, and polyadenylated form, it has to be transported to the cytoplasm for protein translation (Figure 6.1). However, various checkpoints act at the stages of transport and translation in eukaryotes. The first such checkpoint is mRNA stability, with unstable mRNAs being targeted for deadenylation, decapping, and subsequent degradation either in the nucleus or cytoplasm. The machinery that participates in this mRNA decay process acts either in the 5′ to 3′ direction (e.g., the 5′ to 3′ exoribonuclease Xrn1) or in the 3′ to 5′ direction (e.g., the exosome), always after deadenylation (Belasco 2010). Several components of this machinery have been identified in *P. falciparum* using *in silico* approaches (Mair 2010; Shock 2007); nonetheless, their functionality *in vivo* remains to be demonstrated.

Interestingly, an initial characterization of the *P. falciparum* protein interaction network using yeast two-hybrid screens showed that the putative *P. falciparum* mRNA deadenylase PF3D7_0811300 complexes with proteins involved in transcription, chromatin modification, and ubiquitination (LaCount 2005), similar to the mRNA deadenylase-containing Ccr4-Not complex in the budding yeast *S. cerevisiae* (Collart and Panasenko 2012). This hints at a versatile role for PF3D7_0811300, and it also lends support to the idea that deadenylation does not necessarily mean mRNA degradation (Weill 2012) but that regulating polyA tail length may be a novel mode of post-transcriptional, or even co-translational, regulation in *P. falciparum*. Moreover, given that several alternative splicing events in *P. falciparum* asexual stages result in premature stop or nonsense codons (Sorber 2011), such mRNA molecules may never be translated; instead, they may be targeted for degradation by the co-translational nonsense-mediated decay pathway (McGlincy and Smith 2008), the components of which have been identified in *P. falciparum* by bioinformatic analyses (Sorber 2011) and need to be experimentally validated.

The second most common post-transcriptional gene-silencing mechanism found in several eukaryotes, including the kinetoplastid *T. brucei*, is RNA interference (RNAi), which is mediated by short, sequence-specific, noncoding, double-stranded RNAs (dsRNAs such as siRNAs, miRNAs, and piRNAs), the RNAse III enzyme Dicer, and the catalytic single-stranded RNA-binding protein Argonaute (Jinek and Doudna 2009). When the RNAi pathway came to light at the end of the 20th century, malaria researchers scrambled to identify its components in *P. falciparum* by homology searches and experimentation, but with no apparent success (Aravind 2003; Baum 2009; Ullu 2004). Although a few reports described the successful use of dsRNA molecules to achieve gene silencing in *P. falciparum* and *P. berghei* (Malhotra 2002; McRobert and McConkey 2002; Mohmmed 2003), the authors did not provide convincing evidence in support of a classical RNAi pathway in *Plasmodium*. Nevertheless, it cannot be ruled out that the reported RNAi effect could be due to the antisense nature of the transfected dsRNA and not just its general.

In addition to short noncoding dsRNAs, long noncoding RNAs (lncRNAs) and antisense noncoding RNAs (asRNAs) also play a role in post-transcriptional gene regulation (Wilusz 2009), both of which have been described in *P. falciparum* asexual stages using targeted and genome-wide approaches (Broadbent 2011; Epp 2009; Graslund 2008; Lopez-Barragan 2010; Otto 2010; Raabe 2010; Sierra-Miranda 2012; Siegel 2014). But mechanistic insights into their mode of action in

P. falciparum are currently missing. The lncRNAs, as have been identified from the *var* intron (Epp 2009), centromeres (Graslund 2008), and subtelomeric regions (Broadbent 2011; Sierra-Miranda 2012), may act at the chromosome organizational, epigenetic, transcriptional and/or post-transcriptional levels. The asRNAs, which are enriched at the 3′-end of about 44% of *P. falciparum* ORFs (Siegel 2014), may affect nucleosome positioning, affect transcriptional timing, and/or post-transcriptionally downregulate gene expression by sequestering their complementary mRNAs. With the development of high-throughput techniques such as ribosome profiling and NET-seq to study transcription and translation (Konig 2012), ChIP-seq and MNase-seq to study nucleosome positioning (Wei 2012), and the continued discovery of new RNA-binding proteins in *P. falciparum* (see below), this is an exciting time for RNA research in the malaria parasite.

The next checkpoint occurs at the level of translation. Two observations point to the presence of robust translational monitoring in *P. falciparum* blood stages: One is a delay in translation, that is, a delay in the detection of protein relative to its transcript, for a subset of mRNA molecules (~30%) with a median delay time of 11 hours (Foth 2011; Le Roch 2004) (Figure 6.1). The other is a lengthening of mRNA half-life during intra-erythrocytic development, with late schizonts having an average mRNA half-life of about 65 minutes, nearly six-fold higher than early rings (Shock 2007). Together, these findings indicate that once select transcripts are made, either they are maintained in a state of translational repression for a few hours, which may also protect them from mRNA decay, or the corresponding proteins are rapidly degraded and stabilized only when the parasite needs them, supporting a phenomenon of just-in-time translation as opposed to just-in-time transcription. Additionally, given the cascade of gene expression observed during *P. falciparum* asexual stages (Figure 6.1), late-stage mRNAs may have special characteristics, such as *cis*-elements in their 3′-UTR that bind to specific RNA-binding proteins, secondary structure motifs, and so on, that make them more resistant to decay and hence longer lived.

To achieve translational repression, mRNA molecules can be stored (or degraded) in transient multiprotein RNA repression centers in the cytoplasm, such as germ granules, P-bodies, and stress granules (Eulalio 2007), and constituents of such centers have been annotated in *P. falciparum* (PlasmoDB). However, there is no direct experimental evidence supporting the activity of these proteins in *P. falciparum* blood stages, although mRNA-containing translational repression granules clearly exist in *P. berghei* gametocytes (Mair 2006; Mair 2010). In addition, an *in silico* analysis of *P. falciparum* genes found that CCCH-type zinc finger proteins were overrepresented in the parasite genome (Coulson 2004); these proteins are particularly important in regulating mRNA decay and the rate of translation in several eukaryotes, and they couple nucleocytoplasmic events owing to their ability to shuttle between the two compartments and to bind to both DNA and RNA (Sanduja 2011). The identification of PfAlbas (Chene 2012; Goyal 2012), and their localization to the nucleus or cytoplasm in a stage-specific manner further emphasizes the role that dual-specificity proteins play in transcriptional, post-transcriptional, and translational control in asexual stages. Lastly, two members, PfPuf1 and PfPuf2, of the Puf family of RNA-binding translational repressors (Quenault 2011) are expressed in the gametocytes of *P. falciparum* (Cui 2002) and may modulate sexual differentiation (Miao 2010b) by binding to a U-rich element in the 3′UTR of a subset of gametocyte-specific mRNAs and regulating their stage-dependent expression, similar to their *P. berghei* counterparts (Braks 2008). Puf2 also regulates the transition of sporozoites to liver-stage forms (Gomes-Santos 2011; Muller 2011), indicating a key role for this molecule during the *Plasmodium* life cycle.

Upon the successful completion of translation, a protein adopts its three-dimensional folded structure, with or without the help of molecular chaperones, and is ready to perform its function. Yet, it has become clear that there are several post-translational checkpoints that determine the fate of a protein, adding another layer to gene regulation. These comprise chemical and biochemical modifications, transport to different intra- and extracellular compartments, assembly into

macromolecular complexes, proteolysis, degradation, and so on. All of these checkpoints exist in *P. falciparum.* One of the best-studied examples of post-translational control is the modification of histone tails by acetylation, methylation, and phosphorylation; this determines epigenetic regulation (see section on histone modifications above). Other post-translational modifications such as palmitoylation, dolichylation, myristoylation, farnesylation, addition of a GPI anchor, glycoslation, *S*-glutathionylation, ubiquitination, *O*-sulfonation, and sumoylation, have been detected in one to several *P. falciparum* proteins either through whole-proteome analyses or by targeted approaches (reviewed in Chung 2009). However, much remains to be understood about their vitality to parasite development and host–parasite interaction.

Novel gene regulatory mechanisms are constantly being discovered in *P. falciparum*. Two studies have shed light on competitive translational repression and *trans*-splicing as modes of post-transcriptional gene regulation in asexual parasite stages. First, Amulic *et al*. demonstrated that the presence of an upstream ORF (uORF) 360 to 269 bp upstream of the translation initiation site (TIS) in the 5′UTR of the placental malaria-specific *var2csa* gene inhibits translation of *var2csa*, possibly by preventing the ribosome from re-initiating at the *var2csa* TIS (Amulic 2009). Second, Lamonte *et al*. showed that in erythrocytes carrying the sickle cell variant of hemoglobin (HbS), the aberrant integration by *trans*-splicing of two human miRNAs, miR-451 and let-7i, into the 5′-end of essential *P. falciparum* mRNAs such as PF3D7_1223100 and PF3D7_1343000 resulted in an abrogation of their translation and a subsequent impairment of intra-erythrocytic parasite development (Lamonte 2012); this could be one explanation for the inability of *P. falciparum* to develop efficiently in sickle cell heterozygote and homozygote hosts. This study also hints at the possibility that the human miRNAs, including miR-451, that were previously detected in parasite preparations (Rathjen 2006) may be real and not due to contamination from erythrocytic debris or human white blood cells. An elaboration of the role of such miRNAs will open up avenues to understanding host–parasite communication, especially from the viewpoint of the host.

Drug targeting

Gene regulatory mechanisms in the malaria parasite present several promising targets for therapeutic intervention. Currently used antimalarials with known mechanisms of action are either repurposed antibiotics or primarily target heme polymerization and parasite metabolic processes or interfere with the parasite mitochondrion. Alternatively, the molecular target of artemisinin, the key component in combination therapies, is not fully understood. Potential antimalarial targets under investigation include parasite proteases, kinases, and histone-modifying enzymes. These last two target classes act by altering protein post-translational modifications, which, in the context of chromatin, account for a major mechanism of epigenetic gene regulation. The modulation of epigenetic factors by small molecules is most advanced in the field of cancer chemotherapy. Comparing the advances in that field with our knowledge of epigenetic gene regulation in *P. falciparum* provides a basis for evaluating the current status of epigenetics-based antimalarial drug discovery (summarized in Table 6.3) and an outlook for potential future targets of intervention.

Histone acetyltransferases (HATs)

Histone acetylation, generally associated with transcriptional activation, is highly abundant in *P. falciparum* (Dastidar 2012b; Miao 2006; Trelle 2009). The effect of two HAT inhibitors derived from natural compounds, curcumin and anacardic acid, on recombinant PfGCN5 enzyme and *P. falciparum* parasites in culture, has been reported (Cui and Miao 2007; Cui 2008b). Curcumin inhibited recombinant PfGCN5 with an IC_{50} of 48 μM and inhibited parasite growth with an IC_{50} of 20 to 30 μM. Anacardic acid showed a similar effect, inhibiting recombinant PfGCN5 with an IC_{50}

Table 6.3 Predicted and verified histone mark writers in *Plasmodium falciparum*.

Modification	Gene family	Protein *Gene ID*	Validated enzymatic activity?	Inhibitor Compound Series	Cancer Therapeutic?
DNA methylation	m5C MTase	**PfDNMT2** *PF3D7_0727300*			Approved
Histone acetylation	GNAT*	**PfGCN5** *PF3D7_0823300*	(Fan 2004)	Curcumin (Cui 2007) Anacardic acid (Cui 2008b)	Preclinical
	GNAT	*PF3D7_0109500*			
	GNAT	*PF3D7_1437000*			
	GNAT	*PF3D7_1003300*			
	GNAT	*PF3D7_1323300*			
	GNAT	*PF3D7_0805400*			
	GNAT-related	*PF3D7_1227800*			
	MYST	**PfMYST** *PF3D7_1118600*			
	MYST	*PF3D7_0809500*			
	HAT1	*PF3D7_0416400*			
Histone deacetylation	Class I	**PfHDAC1** *PF3D7_0925700*	(Patel 2009)	Apicidin (Darkin-Rattray 1996; Chaal 2010) Hydroxamates (Andrews 2008; Patel 2009)	Approved
	Class II	**PfHDAC2** *PF3D7_1472200*		Apicidin (Darkin-Rattray 1996; Chaal 2010) Hydroxamates (Andrews 2008; Patel 2009)	
	Class II	**PfHDAC3** *PF3D7_1008000*		FR235222 (in T. gondii) (Bougdour 2009)	
	Class III	**Sir2A** *PF3D7_1328800*	(Merrick & Duraisingh, 2007)	Nicotinamide (Prusty 2008); Surfactin (Andrews 2012)	
	Class III	**Sir2B** *PF3D7_1451400*			

(*Continued*)

Table 6.3 (Continued)

Modification	Gene family	Protein *Gene ID*	Validated enzymatic activity?	Inhibitor Compound Series	Cancer Therapeutic?
Histone lysine methylation	SET	**PfSET1** *PF3D7_0629700*		BIX-01294? (Malmquist 2012)	Preclinical (H3K4)
	SET	**PfSET2** *PF3D7_1322100*	(Cui 2008a)		
	SET	**PfSET3** *PF3D7_0827800*			In development (H3K9)
	SET	**PfSET4** *PF3D7_0910000*		BIX-01294? (Malmquist 2012)	Preclinical (H3K4)
	SET	**PfSET5** *PF3D7_1214200*			
	SET	**PfSET6** *PF3D7_1355300*		BIX-01294? (Malmquist 2012)	Preclinical (H3K4)
	SET	**PfSET7** *PF3D7_1115200*			
	SET	**PfSET8** *PF3D7_0403900*	(Cui 2008a)		
	SET	**PfSET9** *PF3D7_0508100*			
	SET	**PfSET10** *PF3D7_1221000*	(Volz 2012)	BIX-01294? (Malmquist 2012)	Preclinical (H3K4)
Histone arginine methylation	Class I (asymmetric)	**PfRMT1** *PF3D7_1426200*	(Fan 2009)	Sinefungin (Fan 2009)	
	Putative Class I (asymmetric)	**PfRMT4/ PfCARM1** *PF3D7_0811500*			
	Class II (symetric)	**PfRMT5** *PF3D7_1361000*			
Histone demethylation	LSD	**PfLSD1** *PF3D7_1211600*			
	JmJC	**PfJmJC1** *PF3D7_0809900*			
	JmJC	**PfJmJC2** *PF3D7_0602800*			
Histone phosphorylation	many potential	many potential			Approved

Note: The final column indicates the stage where the class of protein or human homologue is with regard to cancer therapeutic development: in early-stage development, in pre-clinical investigation, or approved therapeutic.
*GNAT = Gcn5-related histone acetyltransferase.

of 20 μM and parasite growth with an IC_{50} of 30 μM. Acetylation levels of histone H3K9 and H3K14 were reduced with a 12-hour treatment with either compound at 20 μM, whereas levels of H4K5ac, H4K8ac, H4K12ac, and H4K16ac remained unchanged. Anacardic acid treatment also resulted in a two-fold or greater downregulation or upregulation of 207 or 64 genes, respectively, in late trophozoites. Although no inhibition studies were reported for recombinant PfMYST, because this enzyme preferentially acetylates H4K5, H4K8, H4K12, and H4K16, it is likely that curcumin and anacardic acid are specific for PfGCN5 and not PfMYST. It should be noted that curcumin also induces the generation of reactive oxygen species; thus, its cellular effect is likely to involve other factors in addition to its ability to inhibit PfGCN5.

The modest effect of parasite HAT inhibition by curcumin and anacardic acid is comparable to the limited progress of these compounds in cancer chemotherapy (Cole 2008). Instead, the cancer chemotherapy field has benefited from the development of bisubstrate analogues as highly efficacious and specific HAT inhibitors, suggesting that a similar strategy may be required for antimalarial development. In fact, the substrate specificity of HATs is not necessarily limited to histones, which, though a disadvantage for the development of specific epigenetic tools, is advantageous for developing potent antimalarials.

Histone deacetylases (HDACs)

The malaria parasite HDACs have been more amenable to regulation by small-molecule inhibitors. Thus far, of the five PfHDACs, only PfHDAC1 and PfSir2A have been expressed as active recombinant enzymes for *in vitro* inhibition studies (Merrick and Duraisingh 2007; Patel 2009).

The first malaria parasite Class I/II HDAC inhibitor to be described was the cyclic tetrapeptide apicidin (Darkin-Rattray 1996). Apicidin was found to be effective at killing *P. falciparum*, *T. gondii*, and *C. parvum in vitro*, with an IC_{50} of 200 nM against *P. falciparum*. Subsequent inhibition of recombinant PfHDAC1 by apicidin was demonstrated, identifying the likely target of this compound (Patel 2009). Unfortunately, apicidin has displayed poor bioavailability, poor HDAC isoform specificity, and poor selectivity for parasites versus host cells, and efforts to improve on the apicidin scaffold have only been modestly successful (Colletti 2001a, 2001b; Meinke 2000; Singh 2002). Overall, apicidin and its derivatives have proved more useful in basic research applications than for therapeutic use (Andrews 2012; Chaal 2010).

Hydroxamate-based Class I/II HDAC inhibitors have been pursued aggressively for their potential as antimalarials (Andrews 2012). The natural product trichostatin A, one of the first HDAC inhibitors recognized, displayed potent activity against both drug-sensitive 3D7 and multidrug-resistant Dd2 *P. falciparum* strains, with IC_{50} values approaching 10 nM for either strain (Andrews 2008). Other hydroxamates shown to have antimalarial activity include suberoylanilide hydroxamic acid (SAHA, Vorinostat) and sulfonylpyrrole hydroxamate (4SC-201, Resminostat), which are both approved for cancer chemotherapy. Additional derivatives of these parent hydroxamate compounds have been investigated in an effort to improve potency, parasite specificity, and effectiveness in an animal model (Agbor-Enoh 2009; Andrews 2008; Dow 2008; Patel 2009). This series of compounds continues to yield mixed results with regard to host versus parasite selectivity and/or effectiveness in an animal model. Nonetheless, the discovery that the hydroxamate analogue YC-II-88 was able to cure *P. berghei*–infected mice, albeit at 50 mg/kg/day for five days beginning at the time of infection, continues to fuel the pursuit of effective hydroxamate-based parasite HDAC inhibitors for use as antimalarials (Agbor-Enoh 2009).

Class III HDAC inhibitors such as nicotinamide, surfactin, splitomicin, and sirtinol have been tested against recombinant PfSir2A and *P. falciparum* parasites in culture (Merrick and Duraisingh 2007; Prusty 2008). Of these, only nicotinamide inhibited recombinant PfSir2A (Merrick and Duraisingh 2007) and was subsequently found to inhibit parasite growth (Prusty 2008), though at

10 mM in each assay. Because both PfSir2 paralogues, PfSir2A and PfSir2B, have been individually knocked out (Duraisingh 2005; Tonkin 2009), the parasite killing effect of such a high concentration of nicotinamide is unlikely to be specific to PfSir2A inhibition and is more likely due to inhibition of multiple NAD^+-dependent enzymes in the parasite. However, with novel roles for PfSir2A being constantly described (see above), it remains possible that its inhibition *in vivo* could result in parasite death, for which specific inhibitors will need to be discovered.

Histone methyltransferases

Histone lysine methylation is critically important for *P. falciparum* gene regulation and is clearly involved in aberrant gene regulation in human cancer, making HKMT enzymes attractive targets for drug discovery. However, this avenue is only just being exploited. Thus far, only one study has attempted to target histone lysine methylation in *Plasmodium* parasites for antimalarial drug discovery efforts (Malmquist 2012). The known HKMT inhibitor BIX-01294 and one of its derivatives were found to inhibit drug-resistant and sensitive *P. falciparum* growth with IC_{50} values 100 nM or less; these effects were rapid and effective at all stages of the intraerythrocytic cycle. These compounds were also effective in reducing *P. berghei* parasite numbers in patently infected mice, although a single intraperitoneal dose of 40 mg/kg three days after infection did not result in a cure.

In addition to lysine methylation, histones can also be mono- and di-methylated on arginine residues in a symmetric or asymmetric manner. Histone arginine methylation is detectable in *P. falciparum* parasites, and of the three PfPRMTs, PfRMT1 has been expressed as a recombinant protein and its enzyme activity characterized (Fan 2009). Moreover, several small molecule inhibitors of varying specificity toward known PRMT enzymes were shown to inhibit PfRMT1 and *P. falciparum* growth in culture at concentrations near 1 μM, although the specificity of this effect toward histone protein methylation was not determined (Fan 2009). Nonetheless, it may be possible to derive specific inhibitors for PfRMT1 for use as analytical tools or potential antimalarials. Notably, PRMTs are relatively understudied, and as such there is little guidance from other fields to direct their targeting in the malaria parasite.

Histone demethylases

Lysine demethylases have been implicated in various cancers and are current therapeutic targets. Given the importance of histone methylation for gene regulation in *P. falciparum*, and given that there are only three recognizable lysine demethylases in *P. falciparum*, this class of enzymes presents high potential as an unexploited drug target.

Histone kinases

Kinases known to act on histones are active targets in cancer chemotherapy because several of these are mutated or amplified in a variety of malignancies. Indeed, the histone kinase JAK2 and the Aurora family of kinases, which are involved in cell cycle regulation and are implicated in carcinogenesis, are the targets of the chemotherapeutic ruxolitinib and several other small molecules under clinical investigation, respectively (Dawson and Kouzarides 2012). Aurora kinases are responsible for phosphorylating histone H3S10 and H3S28, two marks identified on *P. falciparum* histone H3 (Dastidar 2012b; Treeck 2011). The *P. falciparum* genome encodes three Aurora kinase orthologues, one of which was shown to be essential for parasite survival (Reininger 2011). However, the function of specific histone phosphorylation events and the enzymes responsible for those modifications await more investigation. It may be possible to piggyback on the gains in cancer chemotherapy research to develop parasite-specific Aurora kinase inhibitors as potential antimalarials.

Histone post-translational modification readers

The regulation of chromatin-modifying proteins by small molecules has generally focused on discovering inhibitors of enzymatic activity, as highlighted above. However, there have been several reports of small molecules that inhibit bromodomain-containing BET family members (proteins involved in transcriptional regulation and cell growth and implicated in a variety of cancers) from interacting with their target acetylated lysine residues (Dawson 2012). These inhibitors have proved effective in curing mouse models of various cancers, but they are yet to be tested in humans. The *P. falciparum* genome encodes for at least seven bromodomain-containing proteins, including the HAT PfGCN5 and the putative HKMT PfSET1 (Table 6.4). With the appropriate cellular and biochemical assays, it would be interesting to develop *Plasmodium*-specific inhibitors of bromodomain-acetylated lysine interaction. Indeed, if this strategy proves viable, inhibiting other *P. falciparum* epigenetic readers such as chromodomain-, PhD domain- and 14-3-3 domain-containing proteins will provide novel avenues for potential therapeutic intervention.

DNA methyltransferases

The DNA methyltransferase inhibitors azacitidine and decitabine are used in the treatment of myelodysplastic syndromes and are in clinical trials for treating acute myeloid leukemia. Though azacitidine was found to inhibit the growth of *P. falciparum* 3D7 and Dd2 strains, it did so with relatively high IC_{50} values, close to 1 mM (Andrews 2000). This may reflect weak inhibition of the putative *P. falciparum* DNA methyltransferase orthologue PfDNMT2 or off-target effects, or it may be a result of the inability of the parasite to import pyrimidine analogues.

Perspectives

Although our current understanding of gene regulation in the malaria parasite is far from complete, many aspects of chromatin biology have been unraveled. It is clear that apicomplexan parasites contain orthologues to known eukaryotic transcriptional and epigenetic regulator proteins and that some gene regulatory mechanisms are shared between parasites and other model organisms. Nonetheless, *P. falciparum* also presents some unshared gene regulatory features: histone marks that appear to be used differently in the host versus the parasite – the repressive H3K9me3 mark is restricted in blood stage parasites to clonally variant multicopy gene families – and nonmammalian DNA-binding proteins such as the PfApiAP2s and PfAlbas. This results in phenotypic plasticity in genetically identical parasites and provides a unique regulatory layer that helps *Plasmodium* to adapt to ever-changing host environments, in other words, a bet-hedging strategy. In fact, epigenetically controlled stochastic switching between two phenotypic chromatin states, namely heterochromatin–euchromatin, may control many key events during the life cycle of malaria parasites such as gametocyte commitment, male and female gametocyte development, or hypnozoite formation.

Although *Plasmodium* gene regulation is an active area of research (Cui 2015; Doerig 2015; Josling 2015; Vembar 2014, 2015; Voss 2014) a number of other factors contributing to gene expression have been so far overlooked. For example, the importance of different types of non-coding RNA in chromatin biology and gene regulation is probably underestimated due to the absence of RNAi in *P. falciparum*. We are also just beginning to discover the importance of epigenetic regulation outside the field of *var* expression and immune evasion. Finally, the response of the parasite to host signals, including host miRNAs and host DNA, and to the presence of other pathogens, including other *Plasmodium* species, remains undefined.

Table 6.4 Predicted and verified histone mark readers in *P. falciparum*.

Modification	Domain Family	Protein *Gene ID*	Validated binding?	Cancer Therapeutic?
Histone acetylation	Bromodomain	**PfGCN5** *PF3D7_0823300*		In development
		PfSET1 *PF3D7_0629700*		
		PF3D7_0110500		
		PF3D7_1033700		
		PF3D7_1212900		
		PF3D7_1234100		
		PF3D7_1475600		
Histone methylation	Chromodomain	**PfHP1** *PF3D7_1220900*	(Perez-Toledo *et al.*, 2009; Flueck *et al*., 2009)	
		PfCHD1 *PF3D7_1023900*		
		PF3D7_1140700		
	Chromodomain-like	**PfMYST** *PF3D7_1118600*		
	Tudor domain	**PfTSN** *PF3D7_1136300*		
		PfSMN *PF3D7_0323500*		
	PhD domain	**PfSET1** *PF3D7_0629700*		
		PfSET2 *PF3D7_1322100*		
		PfSET10 *PF3D7_1221000*		
		PfLSD1 *PF3D7_1211600*		
		PfISWI *PF3D7_0624600*		
		PF3D7_1433400		
		PF3D7_1008100		
		PF3D7_1310300		
		PF3D7_1475400		
		PF3D7_0310200		
		PF3D7_1141800		
		PF3D7_1360700		
		PF3D7_1460100		
Histone phosphorylation	14-3-3	*PF14-3-3I* *PF3D7_0818200*	(Dastidar *et al*., 2012)	
		PF14-3-3II *PF3D7_1362100*		

Note: The final column indicates the stage where the class of protein or human homologue is with regard to cancer therapeutic development: in early-stage development, in pre-clinical investigation, or approved therapeutic.

The holy grail of *Plasmodium* biology is developing a definitive cure for malaria. This in turn requires the design of suitable assays for individual steps in gene control that can be used to test the activity of small molecule inhibitors. Specific inhibitors of a key player in *Plasmodium* gene regulation represent not just potential antimalarials but also potentially useful tools for further dissecting the fascinating biology of this parasite.

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CHAPTER 7

Molecular genetic approaches to malaria research

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Our understanding of the basic biology, pathogenesis, and transmission of *Plasmodium falciparum* at the cellular and molecular levels has dramatically improved since methods to transfect parasites were first developed (Crabb and Cowman 1996b; Wu 1995, 1996). Established during the 1990s and refined further ever since, the ability to introduce recombinant DNA into *P. falciparum* and other *Plasmodium* species, particularly those infecting rodents, has enabled us to modify endogenous gene loci to study their functions at different stages of the parasite's lifecycle. Furthermore, the introduction of genes encoding foreign fusion proteins such as those containing green fluorescent protein (GFP) have enabled the visualization of fusion proteins in live parasites. Finally, the ability to modify the expression of essential genes in a controlled manner, the conditional knockdown approach, offers the opportunity to understand the function of essential proteins in the disease-causing blood stage of the lifecycle. These approaches are still being developed, but even as they are they offer a great opportunity to understand the function of proteins that are essential to blood-stage growth, information that will help prioritize these proteins as targets for further drug and vaccine development.

Transfection methods

Transfection methods were originally developed for asexual *P. falciparum* parasites cultured in human erythrocytes. The reason for this is that asexual blood stages can be easily grown and maintained indefinitely, unlike other stages of the lifecycle. DNA plasmids were first introduced into young ring-stage parasites, 0 to 20 hours after infection (hpi), by electroporation using high voltage/ low capacitance (2.5 kV, 25 μF) conditions (Crabb and Cowman 1996a; Wu 1996). Subsequently it was found that low voltage/ high capacitance electroporation conditions (0.31 kV, 960 μF) were more efficient, and this continues to be widely used today (Fidock and Wellems 1997).

An alternative transfection method was later developed in which plasmid was electroporated into uninfected red blood cells (uRBCs) which were then fed to *P. falciparum* parasites, which were able to spontaneously take up the foreign DNA (Deitsch 2001). This result indicated that electroporation of ring-stage parasites probably only works because the parasites take up the DNA from the RBC rather than have it directly enter their cells during the electroporation step. A study on

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the efficiency of the available transfection methods showed that electroporation of uRBCs was 200-fold more efficient than direct electroporation of ring-stage parasites and six-fold more efficient than a combination double-tap method in which electroporated parasites were grown in electroporated uRBCs (Hasenkamp 2012a). These results indicate that the passive uptake of plasmid DNA is probably more efficient than direct electroporation of parasites because the latter severely reduces parasite viability. This is in agreement with an earlier study indicating a 50% to 70% reduction in cell viability based on Giemsa-stained cell morphology of electroporated parasites (Deitsch 2001). In our hands, stably transfected parasites appeared in half the time when fed plasmid-electroporated uRBCs compared to when ring stages were electroporated with the same plasmids (2 vs. 4 weeks).

Apart from using electroporation to deliver plasmid DNA into the uRBCs, it is also possible to transfect *P. falciparum* with polyamidoamine dendrimers, but only with an efficiency similar to or less than that seen with traditional methods, which is perhaps why it has not been widely adopted (Mamoun 1999b).

It is generally agreed that a breakthrough in transfection technology, particularly that which reduces time and cost through automation, could greatly boost our understanding of *P. falciparum* biology. Recent steps in this direction were achieved with full automation of parasite culture and transfection in a 96-well plate format (Caro 2012). Additional procedures for polymerase chain reaction (PCR) analysis of parasites were developed, thus removing the need for parasite culture scale-up and DNA purification. The advantages of this high-throughput approach include being able to simultaneously perform 96 transfections, requiring 20-fold less DNA required for transfection (which can be achieved with small-scale minipreps), a reduced cost due to less culture media and manual time required, up to seven-fold higher transient transfection efficiency, and a 50% to 90% success rate for stable transfections (Caro 2012). Despite these advantages, the equipment required to perform transfection in this way is expensive, predominantly custom built, and unavailable to most labs at this time; however, it may become cheaper and more widely available in the future.

Transient transfection

Transient transfection is a rapid way to genetically modify cells and analyze the phenotype. However, due to the low transfection efficiency in *P. falciparum*, only a fraction (~10^{-5}–10^{-6}) of the parasites actually has the plasmid after transfection (Fernandez-Becerra 2003; O'Donnell 2002). This impairs analyses that involve modifications of gene *loci* or single-cell examinations such as for protein localization. Thus, most experiments are restricted to the detection of high-sensitivity reporter proteins; in *Plasmodium* the most used are chloramphenicol acetyl transferase (CAT) and two forms of luciferase (Luc), which are described below.

The plasmid vector usually has the reporter gene flanked by the transcription regulatory regions such as promoters and terminators and other regions to be analyzed. DNA is transfected by electroporation as described above, and the cultures harvested in 2 to 8 days without drug selection for quantification of reporter activity. Given the variation of transfection efficiency, plasmids harboring mutated or truncated versions of the regions to be analyzed should always be transfected in parallel with a control plasmid, usually the one with full-length wild-type sequence. This kind of approach allowed the identification of the first promoters and terminators that would be used later on to generate the vectors for stable transfections (Crabb and Cowman 1996b; Wu 1995). Interestingly, there seems to be no barrier to the recognition of regulatory regions within the *Plasmodium* genus (Crabb 1997b), although there are differences in promoter strength when transfected in heterologous systems (Azevedo and del Portillo 2007).

Stable transfection

The method of DNA delivery aside, parasites must be able to replicate the plasmid DNA and express the genes it encodes to become stably transfected. The first step in this process is to separate those parasites carrying plasmids from those that do not, and this is achieved by using a drug-selection gene on the plasmid under the control of *Plasmodium* regulatory sequences. The most commonly used positive selection markers in *P. falciparum* are variants of dihydrofolate reductase-thymidylate synthase (DHFR-TS), a protein with dual function in folate biosynthesis and nucleic acid production. The first selectable markers used in *P. falciparum* were mutant DHFR-TS genes taken from *Toxoplasma gondii* (TgDHFR-TS) and *P. falciparum* (PfDHFR-TS) that conferred resistance to pyrimethamine (Crabb and Cowman 1996a; Wu 1996). Both markers were able to maintain stable plasmids episomally or integrated in the genome; however, TgDHFR-TS was preferred because PfDHFR-TS integrates into the native PfDHFR-TS gene locus by homologous recombination.

A major limitation of TgDHFR-TS is that its use is confined to parasite lines that are sensitive to pyrimethamine as well as the ability for parasites to spontaneously develop resistance to pyrimethamine (Thaithong 1992). A mutated human dihydrofolate reductase (hDHFR) that infers resistance to methotrexate and the dihydrotiazine anti-folate drug WR99210 is now the most commonly used selectable marker (Fidock and Wellems 1997; Lewis 1995). WR99210 is a highly specific drug that targets PfDHFR and effectively kills parasites in the nanomolar range against all strains of *P. falciparum,* allowing it to be used in studies using drug-resistant parasite lines (Fidock and Wellems 1997). Because hDHFR is able to replace the function of PfDHFR in parasites and is highly resistant to the effects of WR99210, an increase in IC_{90} greater than 4000-fold is seen in transgenic parasites, making it an ideal selectable marker (Fidock and Wellems 1997).

Several new selectable markers are now available for use in *P. falciparum,* making it possible to perform more-complicated genetic analyses. The blasticidin S deaminase (BSD) gene, from *Aspergillus terreus,* and the neomycin phosphotransferase II (NEO), from transposon Tn 5, confer resistance to the antimalarial drugs blasticidin S and G418, respectively. Both genes target protein synthesis, and both can be used to select for stable episomal or integrated plasmids (Mamoun 1999a). Because both genes encode for enzymes that convert an active drug into a nontoxic product, the increase in IC_{50} for each of the drugs is considerably lower than the difference seen when using hDHFR (Mamoun 1999a; Yamaguchi 1975). This difference in mechanism of action allows the plasmid copy number when using BSD and NEO to be selected for by increasing the drug concentration more readily than with DHFR (Epp 2008). This ability to select for plasmid copy number provides a very simple method for increasing the amount of protein expression from episomal plasmids; however, it requires selection over 2 to 3weeks (Epp 2008). This could be used to assess the target of a drug by overexpressing the target and determining if a decrease in susceptibility is seen. Parasites are able to develop spontaneous resistance to blasticidin S through altered expression of clag 3 (Mira-Martínez 2013; Hill 2007). These changes are associated with altered permeability of the iRBC and usually result in low-level resistance(Mira-Martínez 2013). High-level blasticidin S resistance causes parasites to have a greatly reduced growth rate and therefore will generally be outgrown by parasites containing the BSD selection marker (Mira-Martínez 2013).

The puromycin-*N*-acetyl-transferase (PAC) gene, isolated from *Streptomyces alboninger,* encodes resistance to puromycin, an aminonucleoside antibiotic that targets the large ribosomal subunit and thus protein synthesis (de Koning-Ward 2001; Vazquez 1979). PAC has been used as a positive selection marker to integrate plasmids via single and double homologous crossover into the *P. falciparum* genome (de Koning-Ward 2001). However, the high GC content (73%) of the PAC gene is thought to lead to poor expression in *P. falciparum,* leading to relatively low levels of resistance (de Koning-Ward 2001). For this reason, PAC has not been widely used by the malaria research community.

The most recent positive selection marker, the *Saccharomyces cerevisiae* gene yeast dihydroorotate dehydrogenase (yDHODH), encodes resistance to the antimalarial drug DSM-1 (Ganesan 2011; Ke 2011). DSM-1 is a triazolpyramidine-based inhibitor that specifically targets PfDHODH, an essential protein in the pyrimidine biosynthesis pathway (Phillips 2008). Due to yDHODH replacing the function of PfDHODH, similar to hDHFR, the increase in resistance is much greater than that of BSD and NEO, making it more suitable for gene integration experiments.

Apart from drug-selection markers, parasite-derived DNA fragments inserted into the plasmids themselves can help them be maintained in a stable form. An early example of this is Rep20, a 21-bp repeat from the subtelomeric regions of *P. falciparum.* When inserted into a plasmid, it improves episomal maintenance probably by promoting better segregation between daughter merozoites (O'Donnell 2002). This enables the more-rapid establishment of a stable transgenic parasite population following transfection. More recently, the pFCEN plasmid has been developed, which contains a *Plasmodium* mini-centromere (Iwanaga 2012). It too promotes better segregation and faster recovery of stable transfects, but it has the added benefit that it is maintained as a single copy rather than as a concatamer. This results in more even expression of proteins encoded by the plasmid between individual cells within a transgenic population.

Bxb1 integrase system

Traditional methods for overexpression of recombinant proteins in *P. falciparum* rely on episomal maintenance of plasmids. Uneven segregation of plasmids during replication leads to a reduced growth efficiency because parasites that to do not receive a copy of the plasmid no longer contain a positive selection marker. Episomal plasmids are present in highly variable copy numbers, which results in a very heterogeneous population expressing none, to low, to large amounts of recombinant protein.

For this reason the Bxb1-mediated integration system was developed. This system is based on the ability of the serine integrase of mycobacteriophage Bxb1 to efficiently and specifically recombine an *attP* site with the host *attB* site in a non-reversible manor (Figure 7.1) (Nkrumah 2006). The Bxb1 integrase was chosen because it recombines short *attP* and *attB* sites, which contain regions of inverted symmetry flanked by a conserved 5′ GT dinucleotide in a way that destroys the sites to produce *attL* and *attR* sites and prevents further recombination events and also allows the polarity of recombination to be chosen (Nkrumah 2006). It also requires no divalent cations, cofactors, or DNA supercoiling, making it ideal for relatively simple and rapid integration of plasmid DNA into the *P. falciparum* genome (Nkrumah 2006).

Although the Bxb1 integrase system offers a means of rapid integration of a plasmid into the genome, it does require a number of time-consuming initial steps to generate the necessary plasmids and parasite line. The *attB* site must first be integrated into the genome by classical homologous recombination methods, usually into a nonessential gene (Nkrumah 2006). Once a clonal line containing the *attB* site has been obtained, a single plasmid containing the *attP* site for integration as well as the *bxb1* integrase gene to mediate recombination between the *attB* and *attP* sites can be used. Alternatively, two separate plasmids can be co-transfected: One plasmid will integrate into the genome containing the *attP* site and gene of interest to be expressed, and one plasmid (pINT) will be lost without constant drug selection and contains the *bxb1* integrase gene to mediate integration. By using two separate plasmids, the size of each plasmid is reduced, making the cloning of AT rich *P. falciparum* genes in *Escherichia coli* less difficult. The two plasmid method does, however, require the use of three separate selection markers and extra preparation of DNA for transfection, as well as a slightly reduced efficiency due to the need to simultaneously transfect the same cell with both plasmids.

Parasites transfected with this system can be observed (using the Malstat assay) 14 to 25 days after transfection with a homogenous parasite population containing a single-copy integrated

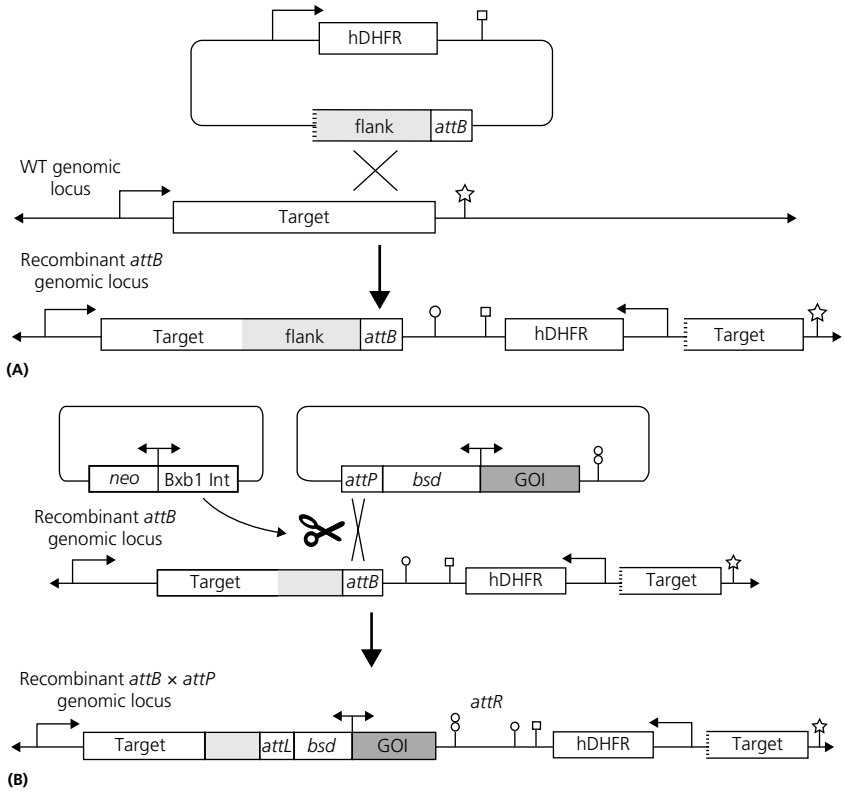

Figure 7.1 *A,* Diagram showing how the Bxb1 mediated integration system works. *B,* The *attB* recombination site is introduced into the wild-type genomic locus of a target gene via single-crossover homologous recombination.

plasmid (Nkrumah 2006). A study has shown that drug selection for the pINT plasmid was disadvantageous because it caused daughter cells that did not receive the pINT plasmid but might contain an integrated target plasmid to die (Spalding 2010). By removing drug selection for pINT combined with using an alternative transfection method (transfecting uRBCs), it is possible to detect parasites an average of 11 days after transfection (0.03% parasitemia, 20 days to reach 1% parasitemia) compared with 15 days when drug selection is applied for pINT (Spalding 2010). This shows that integration occurs rapidly in this system because increasing the time that pINT is present does not increase transfection efficiency.

The Bxb1-mediated integration system has several advantages for expressing recombinant proteins when compared to using episomally maintained plasmids. Most importantly, a homogeneous population containing only a single integrated plasmid copy is achieved rapidly without the need for cloning by limiting dilution. This is important because episomally expressed plasmids often result in a highly mixed population due to uneven copy numbers that lead to some cells expressing very large amounts of the transfected gene while other cells express very little or none of the transfected gene, as was observed using a GFP-tagged line by flow cytometry (Nkrumah 2006). It is also an ideal system for studies outside of the asexual stage of the lifecycle because the stable integrant does not require drug selection to maintain expression, as is the case with episomal plasmids. Expression of recombinant protein using this system has now been shown to be effective for localization of proteins within the iRBC (Melcher 2010; Nkrumah 2006), looking at drug resistance

(Eastman 2011; Ke 2011) and drug sensitivity of gametocytes (Adjalley 2011). It may also be possible to excise the plasmid in the future through over expression of another Bxb1 bacteriophage protein, but this has not yet been shown in *P. falciparum* (Savinov 2012).

Genetic approaches for deriving gene function

Reverse genetic approaches seek to derive a gene's function by modifying the gene and observing phenotypic changes that are informative of genetic function. In malaria parasites, this approach usually involves a targeted disruption or even deletion of the gene of interest. This nearly always takes place in asexual blood stages because it is this stage in which transgenes that target the gene of interest can be introduced. Reverse genetic techniques have provided a vast wealth of knowledge about gene function in malaria parasites, particularly of genes whose functions are not essential for parasite survival in the blood, such as those involved in alternative invasion pathways (Duraisingh 2003a, 2003b; Gilberger 2003; Stubbs 2005; Triglia 2005) and host cell modification (Maier 2008). Reverse genetics has also been exceedingly useful for understanding the functions of genes that are expressed in other stages of the parasite's life cycle, particularly those in the mosquito.

Single crossover integration

Single crossover transgenics is by far the simplest approach to disrupt a gene in *P. falciparum.* A single fragment derived from the gene of interest is inserted into a transfection plasmid upstream of a drug-selectable gene cassette, and once taken up by the parasite it potentially inserts itself via homologous recombination into its target gene. To have the greatest chance that disruption of the gene will produce a nonfunctional protein, the targeting gene flank should integrate as close to the start codon as possible. This need, though, has to be balanced with having to make the integration flank as large as possible (>0.8 kbp) to improve the chances of homologous targeting. In some instances, particularly with small genes, in might be necessary to use regions of the 5′ UTR as part of the targeting flank. One drawback here is that noncoding regions in *P. falciparum* are particularly A+T-rich, making their amplification and cloning in *E. coli* more difficult.

Once introduced, the integration plasmid is maintained as a multicopy episome by the parasite as well as integrating into the locus of interest or even elsewhere in the genome. To select for chromosomal integrations over episomal copies, the selectable drug, usually WR99210, is removed for a few weeks and then reintroduced. Episomes are lost from about 20% to 30% of parasites per generation (O'Donnell 2002), and by reapplying drug selection several generations after removing it, parasites with chromosomal copies of the drug-resistance gene cassette can be selected over those that have lost their episomes. Three or four drug selection cycles may be required to produce a parasite population with mostly chromosomally integrated plasmids.

Although the homologous gene targeting is normally favored in *Plasmodium* species, integration at other loci can occur, particularly if disruption of the coding sequence of the gene of interest is deleterious for parasite growth. To isolate potential gene-knockout parasites from other drug-resistant parasites in the transfected population, single clonal populations are recovered by limiting dilution. Once clonal populations are recovered, disruption of the gene of interest can be verified by PCR and/or Southern blot analysis. Western blot analysis with and antibody to the gene of interest is also highly desirable.

The first published account of targeted gene disruption in *P. falciparum* was of the gene for knob-associated histidine-rich protein (KAHRP) (Crabb 1997a). This protein is exported into the host erythrocyte, where it accumulates in clusters below the plasma membrane of the erythrocyte, forming knob-like structures to which the surface-exposed cytoadherance PfEMP1 proteins are anchored. The loss of KAHRP ablated knob expression and substantially reduced cytoadherance.

Single crossover integration as a method for gene knockout has largely lost favor to the double-crossover integration method (discussed below), but nonetheless it remains extremely useful for appending tags onto the C-terminal ends of proteins (see section on protein reporters below). Another area where single crossover integration is still a rewarding approach is to knock down but not ablate gene function by truncating the protein of interest. This was illustrated for RAP1, an essential rhoptry protein that in some parasite lines can be C-terminally truncated to produce a shorter protein that is unable to chaperone other members of the RAP complex to their correct destinations (Baldi 2000). Even truncations of nonessential proteins such as Pf332 can provide useful information about protein function. Pf332 is the largest known exported protein and is associated with Maurer's clefts and the erythrocyte cytoskeleton (Nilsson 2012). Ablation of the protein's gene produces no measureable phenotypes, but truncation changes stiffness of the plasma membrane of the infected erythrocyte (Hodder 2009).

Double-crossover integration

In double-crossover transgenics, two targeting flanks each matching a different end of the gene of interest are placed on either side of the drug selection cassette. The goal here is to completely delete the gene of interest by double site recombination, thereby eliminating the possibility of the gene-disruption plasmid looping out and restoring the original locus. This can be a problem with single-site gene disruptions when drug selection is removed. The other advantage of double-crossover transgenics is that the plasmid backbone is not introduced into the chromosome, which itself can become a target for recombination if the parasites are transfected with a second plasmid.

One drawback of the double-crossover approach is that it can be difficult to isolate parasites with double-site integration from parasites in which the transfection plasmid has integrated by only one of the two sites. To overcome this, plasmids have been designed with an additional negative-selection marker outside of the integration flanks. To date, two negative-selection proteins have been used. First was herpes simplex thymidine kinase (TK), which conferred susceptibility to the compound ganciclovir, which is phosphorylated by the enzyme to a compound that terminates DNA synthesis. TK has now largely been replaced by cytosine deaminase/uracil phosphoribosyl transferase (CDUP) from *Saccharomyces cerevisiae*, which converts 5-fluorocytosine (5FC) to 5-fluoruracil, which, after further conversion, inhibits DNA synthesis as well. CD-containing double-site recombination plasmids have gained favor because they appear to kill CD-expressing parasites faster and produce fewer resistant parasites than TK. Selection against single-site integrants and episome-containing parasites begins when ganciclovir and 5FC are added once drug cycling for integration commences.

Although each knockout parasite line takes several months to make, it is possible to attempt many knockouts at once and perform large-scale functional screens. The largest and most successful of these screens was performed by Maier's group (2008). This screen targeted 83 genes; 51 of these genes were known or predicted to be exported into the erythrocyte, mostly because they contained a Pexel export signal. The remaining genes encoded blood-stage proteins not predicted to be exported. Overall, 53 of 83 genes (64%) could be deleted, with only 24% of the exported proteins being essential, about half the rate of the other proteins. Examination of the knockouts revealed some interesting phenotypes. Six mutants could not display PfEMP1 on the erythrocyte surface due to defects in trafficking the cytoadherance protein. Two of the mutants were defective in knob formation, and four had increased host cell membrane rigidity.

Knockin mutations

Single-crossover integration approaches can also be used to knock in targeted amino acid mutations in parasite proteins to study their function. An example of this is the parasite protein kinase G (PKG), which can be inhibited by compound 1 (McRobert 2008). A small threonine residue in the gatekeeper position is replaced with a bulkier glutamine residue; this blocked compound 1's access

to the kinase's ATP-binding pocket. This amino acid change reduced the compound's inhibitory effects upon gametogenesis, thereby validating PKG's role here (McRobert 2008).

Engineering genetic mutations

Recently, methods for rapidly engineering precise changes into gene loci have been developed. The first trialed for *P. falciparum*, involves zinc finger nucleases (ZFNs), which induce double-stand breaks in the genome and trigger homology directed repair. Rapid insertion of the targeting construct into either an integrated copy of green fluorescent protein or the *Pfcrt* gene to produce specific point mutations that confer parasite resistance to chloroquine, could be obtained with and without direct selection within two weeks, as only homologous end joining occurs in *Plasmodium* (Pradhan 2005, Straimer 2012). The genome editing methodology termed Clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated protein (Cas9) system (CRISPR-Cas9) from *Streptococcus pyogenes* (Mali 2013) has also recently been adapted for *P. falciparum* (Ghorbal 2014). CRISPR was used to disrupt the knob-associated histdine rich protein (*kahrp*) as well as make single nucleotide substitutions in the origin of recognition complex 1 (*orc*) gene (Ghorbal 2014). In addition, the CRISPR-Cas9 system was used to reproduce a polymorphism associated with slow-clearing parasites in malaria patients treated with artemisinin (Ghorbal 2014). Specifically a mutation was introduced to into the propeller region of the kelch protein Pf3D7_1343700 (Straimer 2015). Although the CRISPR-Cas9 system utilizes a simpler approach to the ZFNs, it is more cost-effective and efficient, and thus it is likely that this approach will become the method of choice and may ultimately replace conventional techniques to generate specific gene knockouts and to make single-nucleotide substitutions in genes to decipher biological function.

Forward genetic screens

Forward genetic screens begin by creating a library of parasites with random genetic mutations. These libraries can then be screened for the phenotype of interest and the faulty gene responsible identified. Mutagenesis by transposon insertion, radiation, and chemicals has been widely used in many organisms, but in *Plasmodium,* transposon tagging offers the most promise particularly the *piggyBac* system, which has now been adapted to human and rodent parasite species (Figure 7.2) (Balu 2005; Fonager 2011). The *piggyBac* transposon, derived from the cabbage looper moth *Trichoplusia ni,* integrates into TTAA sites, which are extremely common in A+T-rich *Plasmodium* genomes, ensuring integration is almost random.

To produce a *P. falciparum* transposon–tagged library, the hDHFR drug selection cassette was inserted into a plasmid flanked by the long terminal repeats (LTR) of the *piggyBac* transposon (Balu 2005). On another plasmid was the *piggyBac* transposon under the control of *Plasmodium* regulatory sequences. Both plasmids were electroporated into erythrocytes, and these were infected with parasites, which took up the plasmids. The transposase enzyme inserted the LTR-flanked drug-selection cassette into chromosomal TTAA sites, and these parasites were selected with WR99210. Because the transposase plasmid has no selectable marker, it was lost from the parasite population after doing its job. The analysis of a selection of 123 single clones isolated by limiting dilution indicated the growth rates of almost half the parasite clones were significantly reduced (Balu 2010). The *piggyBac* integration sites were identified by inverse PCR and were found to reside in coding as well as non-coding flanking sequences. Slow-growing phenotypes were due to insertions a range of functional gene categories, with those coding for RNA binding proteins being overrepresented. This probably indicates the importance of post-translational gene regulation for blood-stage parasite growth (Balu 2010). One drawback of this system is that *piggyBac* insertions that inactivate essential blood-stage genes and produce nonviable parasites cannot be studied.

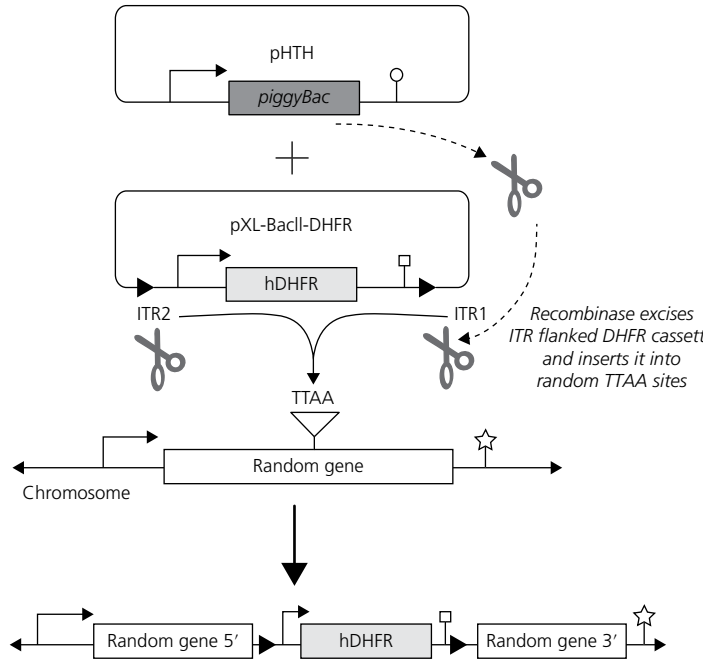

Figure 7.2 Diagram outlining how the *piggyBac* transposon system works. Parasites are fed erythrocytes electroporated with the pHTH and pXL-BacII-DHFR plamids, which are then taken up by the parasites. Expression of the *piggyBac* transposase (scissor symbol) leads to excision of the long terminal repeat (LTR)-flanked DHFR cassette and insertion into random TTAA sites within *Plasmodium* chromosomes. Insertions can occur within or flanking protein -coding sequences. After obtaining a library of parasite clones, the *piggyBac* insertions and their flanking genes responsible for interesting phenotypes can by identified by PCR.

Conditional knockdown of protein function

Although gene knockout can be achieved through homologous integration by single or double crossover, such approaches are not applicable to genes that are essential to the intraerythrocytic cycle. In order to regulate the expression of essential proteins at functional levels, inducible systems have been developed. The methods to specifically target and/or regulate DNA sequences, RNA levels and stability, and protein stability are described below.

Cre/loxP recombinase: Deletion of DNA sequences

Site-specific Cre recombinase can be used to delete sequences in the genome that are flanked by 34-bp sites called *loxP*. Introduction of the recombination sites is done through double crossover, as described above, and the transgenic line is further transfected with the pCre/bsd vector, allowing expression of the recombinase (Figure 7.3A) (O'Neill 2011). Full deletion of the target sequence is achieved and the episomally maintained pCre/bsd vector can be quickly lost through removal of blasticidin. A comparison in *P. falciparum* of the Cre/*loxP* system and a similar FLP recombinase system that acts upon FRT sites and has been used in *Plasmodium berghei* indicated that the former worked more efficiently (O'Neill 2011). Attempts to conditionally regulate the expression of the Cre recombinase through use of the tetracycline-regulatable gene-expression system were unsuccessful

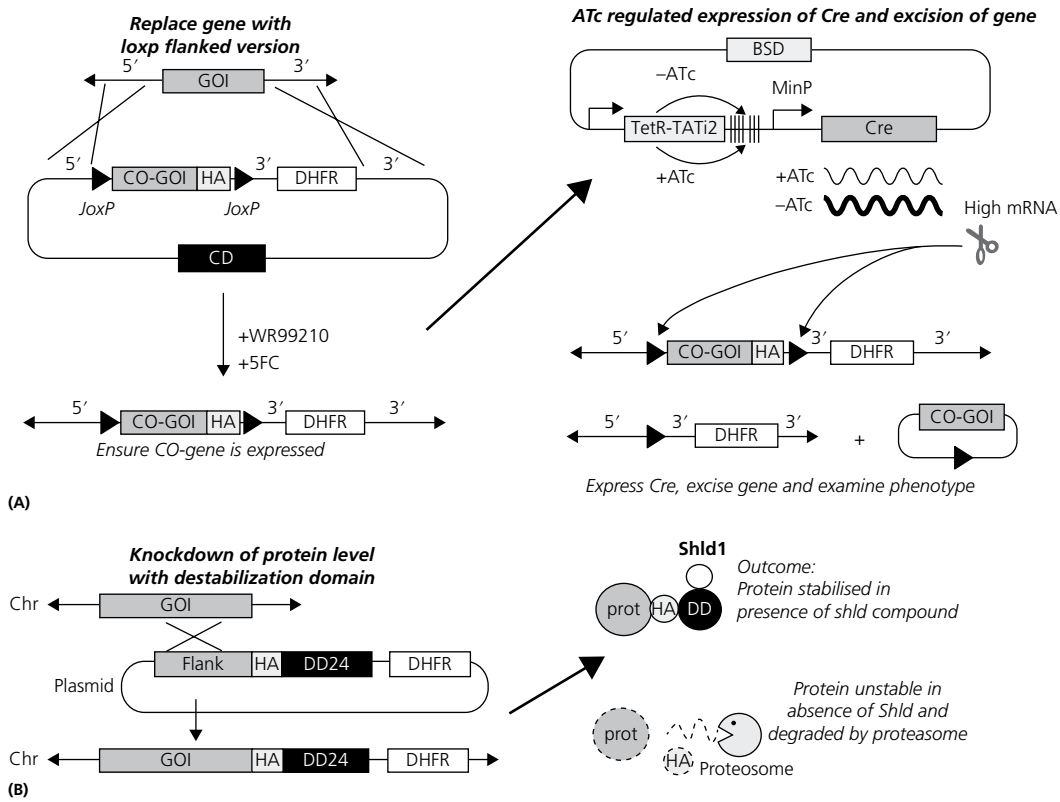

Figure 7.3 Diagram showing how conditional Cre/*loxP* DNA deletion and protein destabilization systems work. *A,* The gene of interest (GOI) is replaced via double site recombination with another version of itself encoding the same amino acids but with a different codon usage. This codon-optimized gene of interest (CO-GOI) is flanked with *loxP* recombination sites and is contained on a gene cassette also containing DHFR selectable marker. The CO-GOI is epitope tagged (HA) so its expression can be verified in place of the original gene. These parasites are then transfected with a Cre recombinase conditionally expressed to ensure deletion of the CO-GOI only occurs when desired. In the example shown here, Cre is under the control of an anhydrotetracycline (ATc) regulated promoter. In the presence of ATc, the TetR-TATi2 transactivator protein cannot bind to the *tet07* repeats (vertical bars) preceding the minimal promoter (MinP), leading to weak transcription of Cre. Once ATc is washed out of the parasites, TetR-TATi2 binds to *tet07* recruiting transcription factors, which results in strong transcription of Cre. The recombinase can then excise the CO-GOI, producing a phenotype diagnostic of gene function. *B,* To conditionally regulate degradation of a protein encoded by a gene of interest, the GOI is tagged with a DNA encoding an epitope-tagged destabilization domain 24 mutant (HA-DD24). The HA-DD24 DNA is fused to a gene-targeting flank and replaces the 3′ end of the GOI via single site recombination. During the transfection and integration period, Shld-1 compound is added to stabilize the GOI-HA-DD24 tagged fusion protein. Once the desired parasites expressing the fusion protein have been cloned, Shld1 is washed out and the parasites are examined for a phenotype due to degradation of the GOI-HA-DD24.

due to unacceptably high levels of basal excision, probably caused by low background levels of recombinase expression (Figure 3A) (Meissner 2005; O'Neill 2011).

The importance of being able to better conditionally regulate Cre expression cannot be overstressed, because this will be required to examine the functions of *loxP* flanked essential genes. Cre function has to be negligible during the transfection period to prevent premature deletion, and it

has to be extremely high when activated to produce a parasite population with greater than 90% gene loss for phenotypic analysis. Although some of the systems listed below might be able to achieve this, a ligand-dependent dimerizable Cre recombinase adapted for *T. gondii* offers real promise (Andenmatten 2012).

Tet system – conditional transcriptional regulation

Regulation of gene transcription by tetracycline-derived operons has been adapted to eukaryotic cells for nearly two decades. An advantage of the system is the high-affinity interaction of the tetracycline repressor (*tet*R) and its operator sequence (*tet*O). Regulation of transcription in eukaryotes happens mainly through activation, and so the TetR was fused to a viral activation domain, creating a transactivator (tTA). Binding of tTA to a minimal promoter activates transcription, which can be shut off by adding a tetracycline analogue, which binds to tTA, changing its conformation so that it no longer binds to *tet*O (Figure 7.3A).

The evolutionary distance of apicomplexa parasites required new activator proteins to be found so they would be able to interact with parasite transcription machinery. The model apicomplexan organism for reverse genetics, *T. gondii,* was used to screen for a new transactivator (TATi2), which was also functional in *P. falciparum* (Figure 7.3A) (Meissner 2002, 2005). The system has allowed the expression of toxic proteins, in which the expression was kept silent by cultivating the transfected parasites in the presence of anhydrotetracycline (ATc). Removal of the drug quickly induced expression, which was used to identify MSP1 regions required for membrane anchoring and trafficking to the plasma membrane (Gilson 2008).

The system proved useful for protein localization, but the transactivator had a toxic effect and was only tolerated when expressed at schizont stages. This is probably because high and constant levels of transactivator bind and sequester transcription factors, interfering with normal gene regulation. This effect is called *squelching* and can to an extent be overcome by restricting transactivator expression to only specific stages of the cell cycle or by conditionally expressing it (see below). In addition, inducible expression was only achieved in about 15% of the cells and the levels were never very high, which precluded analysis of dominant negative phenotypes (Meissner 2005).

Riboenzymes: Conditional transcript degradation

Regulation of transcript levels can be achieved by expressing autocatalytic RNA in *cis* with the gene of interest, which causes a self-cleavage of the mRNA, decreasing its stability. The principles of such a system have been established in *P. falciparum* and in *T. gondii;* however, the inhibitor was ineffective at preventing self-cleavage in *P. falciparum* and too toxic to be of practical use in for *T. gondii* (Agop-Nersesian 2008).

FKBP DD system: Conditional protein degradation

Altering protein levels by directly regulating their turnover offers an advantage over the pre- and post-transcriptional regulation methods described above because one does not have to wait for the protein already synthesized to be naturally degraded. One of the most universal protein-destabilization systems is based on a mutant form of the human FKBP12. This mutant, called a destabilization domain (DD), is expressed fused to the protein of interest at its C or N terminus and targets the whole protein for degradation. Adding a nontoxic, cell-permeable ligand called Shld-1 prevents degradation, and so protein levels can be controlled (Banaszynski 2006).

The DD system has been adapted for *P. falciparum* (Armstrong and Goldberg, 2007), and the roles of a calpain and the calcium-dependent protein kinase 5 (CDPK5) in the cell cycle and egress from

erythrocytes, respectively, was revealed (Russo 2009; Dvorin 2010). The main approach of these studies consisted of integrating a plasmid into the endogenous locus of the gene of interest in a way that the protein would be expressed as a fusion with DD at its C-terminal (Figure 7.3B). The whole process is done in the presence of Shld-1, so the DD fusion is kept stable. Removal of Shld-1 promotes rapid protein degradation to levels where it does not fully perform its physiological function.

We have tried to DD tag more than 10 different proteins and could not get a conditional phenotype for most of them. Some plasmids would just not integrate, suggesting the protein is not functional with the DD tag at its C-terminus. For other proteins, integration was successful but would not produce a phenotype, suggesting the degradation was not low enough and/or that low expression was tolerated by the parasites (Azevedo and Gilson, unpublished experiments). DD mutants with better regulation have been identified (Chu 2008), and they appear to perform better in *P. falciparum,* too (de Azevedo 2012). With improved regulation it may be possible to obtain conditional knockdown at the functional levels for a greater range of proteins.

Another approach that has not been explored with the DD system is the expression of proteins that could produce a dominant negative phenotype from episomes. An advantage of this strategy is that it does not require a long period of culturing to integrate the DD plasmid into its target locus.

DHFR DD system: Conditional protein degradation

A system very similar to the one based on the FKBP12 was further developed based on mutants of the *E. coli* DHFR protein (Iwamoto 2010). The ligand used to stabilize the DD-tagged protein is the drug trimethoprim (TMP), and therefore two proteins can be independently regulated in the same cell, one with Shld-1 and the other with TMP. Although the system works in *P. falciparum* (Muralidharan 2011), it requires parasites to be already transfected with hDHFR-based vectors because TMP inhibits the DHFR of *P. falciparum*.

Aggregation domain: Conditional blockage of protein trafficking

Protein function can also be regulated by conditionally regulating the protein's capacity to traffic properly. A system where protein can be reversibly aggregated in the ER, which prevents secretion, has been established (Rivera 2000). Similar to the DD systems, the protein of interest is expressed as a fusion with the conditional aggregation domain (CAD), which causes it to aggregate in the ER compartment, preventing it from being secreted. The process is reversed by the ligand AP21998 (Ariad Pharmaceuticals, Cambridge, MA, USA). An adaptation of this system to *P. falciparum* showed good control of exported GFP trafficking and also of the nonessential protein PfSBP1 (Saridaki 2008). However, no essential protein has been studied using this system.

Protein reporters

Fluorescent proteins and enzymes have been used as reporters in *P. falciparum*. Given the low transfection efficiency, fluorescent proteins require stably transfected parasites, and enzymes are suitable for both transient and stable transfections.

Localization tags

The location and timing of expression of a protein within an iRBC can provide valuable information about its role in the parasite's lifecycle. As an alternative to raising antibodies against a protein, which can be time consuming and expensive and might produce cross-reactive or non-reactive antibodies, proteins can be tagged with an epitope or fluorescent tag to determine their location within an iRBC. Green fluorescent protein (GFP) was the first of these proteins to be expressed in

P. falciparum (Van Wye and Haldar 1997). In a stream of literature too extensive to review here, there have now been hundreds of published examples of GFP fusion proteins being expressed in *Plasmodium* species, and the approach has become a cornerstone of *Plasmodium* molecular biology.

Apart from its use as a localization tool, GFP has been useful for inferring aspects of parasite development and cellular organization. For example, when GFP fused to the COPII protein Sar1p and was laser-bleached in a small region of developing schizonts, recovery was rapid, suggesting that the ER in young developing merozoites is interconnected (Adisa 2007).

Fluorescent proteins now come in a variety of colors, enabling co-expression with different fusion partners (Jirage 2010). Fluorescent proteins with other useful properties are now also being used. For example, the photo-switchable protein Dendra2, which can be converted from green to red fluorescence by exciting with a 405 nm laser, was used for time-course imaging live cells and showed that protein trafficking to Maurer's clefts continues long after the clefts have moved into the erythrocyte cytosol (Gruring 2011).

However, the large size of fluorescent proteins can lead to disruption of protein function and localization (Knuepfer 2005). For this reason small epitope tags, most commonly a 3× repeat of the nine-amino-acid hemagglutinin (HA) epitope tag, are commonly used, particularly when tagging the native gene locus, where it is more important to maintain normal protein function and expression. The disadvantages of using epitope tags are that they do not naturally fluoresce and require time consuming immunolabeling, and the fixation process can alter cellular morphology, which can lead to nonspecific artifacts.

Alternatives to fluorescent proteins and immunological epitopes now exist that allow protein localization to be visualized using fluorescent dyes. One such system is the tetracysteine (TC) tag. The TC tag consists of a short 6-amino-acid tetracysteine peptide (CCXXCC), making it more suitable than large fluorescent proteins that may interfere with the normal function and/or localization of a tagged protein (Knuepfer 2005). Membrane-permeable biarsenical fluorophores (BAFs), such as FlAsH and ReAsH, preferentially bind to this motif, allowing direct labeling of parasite proteins in live or fixed cells and allowing pulse chase experiments using two different dyes (Crivat 2011). To our knowledge, this system has only been used to determine the localization of a KAHRP construct (Crivat 2011). Another advantage of this system is that some TC reagents can be used to directly label the target protein for electron microscopy. However, the relatively high abundance of cysteine-rich proteins in *Plasmodium,* a relatively low signal-to-noise ratio, and more-rapid photobleaching than GFP will most likely minimize the effectiveness of this system (Crivat 2011).

A relatively new tag is the HaloTag, a common tool in mammalian cells (Los and Wood 2007). The HaloTag (Promega, Fitchburg, WI, USA) is a recombinant catalytically inactive derivative of a bacterial hydrolase (Los and Wood 2007). HaloTag has many advantages over traditional fluorescent tags, because it is highly versatile due to its ability to covalently bond to a reagent that can be linked to a large variety of functional molecules, particularly fluorescent dyes and protein-purification reagents. This allows a single tag to be used for live and fixed cell imaging, pulse chase using two separate fluorophores, specific visualisation on SDS-PAGE gels using a fluorescent scanner, and protein purification. Due to its relatively large size, however, the HaloTag may suffer from the same problems seen with fluorescent proteins. To our knowledge it has only been used to determine the localization of one *P. falciparum* protein, the gametocyte protein Pfg27, although it may become more commonly used in the future (Camarda 2009).

Luciferases

Given their high sensitivity, luciferase enzymes were the first reporters used to detect protein expression in transiently transfected *Plasmodium* parasites. Chloramphenicol acetyl transferase (CAT) was initially used to characterize the minimal regions capable of acting as promoters and

terminators (3′UTR) in *P. falciparum* (Crabb and Cowman 1996b; Wu 1995), followed by firefly luciferase (Luc), which required a simpler assay (Horrocks and Kilbey 1996) and is still the most used nowadays. Other luciferases, such as the Renilla system (Promega) (Militello and Wirth 2003) can be used together with firefly Luc as a normalization control for transfection efficiency.

Parasites are transfected as described above and harvested 2 to 4 days later. Since the assay measures light, which is blocked by hemoglobin, treating iRBC with 0.15% saponin and then washing twice with PBS is highly recommended and increases the signal detected. Promoter characterization, including identification of *cis* regulatory elements, has been extensively analyzed by this method, which has also served to establish and test inducible expression systems. Stable transfected parasite lines expressing luciferase have been generated, and due to the high expression, only a few hundred parasites are needed for detection (Fernandez-Becerra 2003). Therefore, removal of hemoglobin can be bypassed and infected erythrocytes lysed straight in lysis buffer (Hasenkamp 2012b), which renders the assay more suitable for high-throughput approaches such as drug screenings (Cui 2008).

Conclusions

Transgenic approaches continue to have a strong future for resolving gene functions in *P. falciparum* and other *Plasmodium* species. To date, much has been revealed about the roles of non-essential blood-stage genes as well as genes that are expressed at other lifecycle stages. If substantial improvements in the range and effectiveness of conditional genetic knockdown approaches can be made, they would offer real future promise for being able to discover the functions of essential blood stage genes on a large scale. To achieve this will require improved transfection methods that yield transgenic parasites more quickly and permit transfection of linear DNAs, in particular in *P. falciparum*. Linear DNAs can only be maintained if they integrate into their target genes soon after transfection and should facilitate the faster recovery of integrated parasites, important for making conditional knockdown lines. Improved reporter proteins for drug screening, particularly those that can report the enzyme targets of the drugs or other effects they are having on the parasite's biology, are highly desired. The combination of improved functional understanding of parasite proteins along with better screening methods should accelerate future options for improved drug discovery.

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CHAPTER 8
Transcriptomics and proteomics

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The early 21st century can be considered the golden age of genome sequencing. The availability of a vast array of different genomes, including a number of different *Plasmodium* species, has changed our understanding of parasite biology. This availability of massive amount of new genetic information has not only been of immense value but also has provided new challenges. In particular, it became clear that knowledge of the genome sequence alone is not sufficient to fully understand biological processes. This is because genome information is a static insight into the coding capacity of an organism. Hence, besides the gene sequence, it is imperative that we characterize the dynamic and temporal use of the genetic information during the life cycle of an organism in order to fully understand the use of its genetic information. Moreover, it will be important to gain full understanding of the genome related to the complex life cycle of the parasite involving multiple hosts and stages as well as the fact that a large number of predicted parasite genes have no known homologues and therefore no known function.

Transcriptomics refers to the studies of global patterns of the complete collection of RNA molecules within complex biological systems such as individual cells, tissues, and microbial populations. The main underlying principle behind transcriptomics is to define gene expression and transcriptional patterns that reflect physiological states of cells within the given biological system that corresponds to queried conditions such as developmental processes, cell and life cycle progressions, or responses to external conditions. The goal of transcriptomics is not only to identify key gene factors but in its final stage to define complex interactions within the biological network that is connected to the overall network of biochemical, cellular, and other types of physiological processes associated with each biological system. Analogously, the key principles of proteomics follow the same reasoning, with the main focus on total complex of protein expression within the biological system in both qualitative and quantitative manners.

Given the objective limitations of currently available techniques for both, combination of transcriptomics and proteomics is often used as a powerful approach for characterization of complex biological systems. This is achieved by employing high-throughput approaches in order to generate large-scale datasets using the most advanced analytical technologies including DNA microarrays and new generation sequencing (NGS) for transcriptomics and mass spectroscopy-based protein detection for proteomics. Due to the nature of these methodologies that generate large-scale datasets capturing genome- and proteome-wide expression profiles, for the final interpretations, transcriptomics and proteomics is intimately linked with an integrative bioinformatics analysis, also known as systems biology.

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With this rationale, transcriptomics, proteomics, and systems biology studies have been vigorously pursued in studies of malaria parasites (*Plasmodium* species), focusing on several crucial aspects of *Plasmodium* biology. Naturally, these genome-wide and proteome-wide studies rely on the availability of *Plasmodium* genome sequence that started to emerge in the early 2000s. The first genome drafts of *Plasmodium falciparum* (Gardner 2002) and *Plasmodium yoelii* (Carlton 2002) were published in October 2002. Genome sequencing has been accomplished for five additional *Plasmodium* species, including another important human pathogen, *Plasmodium vivax* (Carlton 2008), the rodent models *Plasmodium chabaudi* and *Plasmodium berghei* (Hall 2005), and the macaque pathogen *P. knowlesi* (Pain 2008), which is also capable of human infection (Singh 2004). The genome sequence data along with annotation, and, gene and protein expression information, is publicly available in databases such as PlasmoDB and GeneDB, both of which are currently the most important and comprehensive data source for all *Plasmodium* genomics (Kissinger 2002).

Sequencing of *Plasmodia* genomes has generated a considerable amount of information regarding their transcriptome and proteome. These studies could be broadly categorized into two general foci: fundamental biological processes associated with the life cycle progression and regulation, and physiological plasticity of *Plasmodium* populations defining its adaptation to external conditions. In this chapter we summarize these studies, describing the dynamic properties of the *Plasmodium* genomes that have brought great progress for scientists across the world in understanding the malaria parasite's biology in finer detail.

Transcriptional profiling throughout the parasite life cycle

Intraerythrocytic developmental cycle

The first transcriptomics analyses were triggered with the completion of the first complete genome sequence of *P. falciparum* in 2002, which revealed the presence of about 5300 genes across 14 chromosomes, with one of the lowest G+C contents (19%) among the genomes sequenced up to that time (Gardner 2002). The transcriptome studies in *P. falciparum* were initiated simultaneously using microarray analyses. The first focus was the progression of the *Plasmodium* life cycle in order to understand the extent and relevance of stage-specific gene expression that was suggested by numerous single-gene studies in the past. These first microarray-based genome-wide analyses have not only confirmed these predictions but also exceeded the general expectations suggesting that essentially every gene in the *Plasmodium* genome is under stage-specific transcriptional control.

First, the transcriptome analysis of the intraerythrocytic developmental cycle (IDC) that was carried out with high-density sampling of highly synchronized *P. falciparum* parasites revealed that this asexual blood-stage development is characterized by a transcriptional cascade involving essentially all genes utilized during this phase of the life cycle (Bozdech 2003). Essentially, all genes encoding enzymes and proteins of the IDC cellular functions (>60% of the genome) are transcribed in a coordinated fashion at the precise timing of their requirement in the cell. The periodicity in the expression pattern of genes followed bell-shaped transcriptional profiles indicating just-in-time expression. This time-dependent expression of genes defines the evolution of specialized modes of transcriptional regulation, which includes both conserved cellular processes as well as *Plasmodium*-specific functionalities. A concurrent study that characterized both the intraerythrocytic and the exoerythrocytic developmental stages including sporozoites and gametocytes showed that 88% of the parasites' genes are expressed and 49% of genes are regulated at the transcriptional level in at least one developmental stage of the *P. falciparum* life cycle (Le Roch 2003). Both studies have shown that, while basic cellular function genes are expressed predominantly in the fast-growing stages (trophozoites and early schizonts), genes involved in parasite-specific functions such as

host–parasite interactions and invasions are actively transcribed in the invasive forms (late schizonts, merozoites, and sporozoites).

These earlier studies have been complemented by direct sequencing of expressed RNA transcripts (RNA-Seq) using next generation sequencing (NGS) technologies (Otto 2010). Analyzing the global transcriptional profiles at seven time points across the IDC, a total of 4871 transcripts, which account for more than 90% of *P. falciparum* genes, were found to be expressed and transcriptionally regulated. This result brought the final appreciation for the extent of stage-specific transcriptional regulation, suggesting that a vast majority (if not all) of genes in the genome are expressed (or suppressed) at specific sections of the *Plasmodium* life cycle based on the requirements for their function. In addition, the RNA-Seq data helped to correct about 10% (423) gene models predicted by the original genome sequencing project, and 121 genes were newly discovered. These RNA-Seq data confirmed up to 75% of the predicted splicing events in *P. falciparum* genes, and also confirmed previous suggestions of paucity of alternative splicing during the IDC. Specifically, only 84 putative alternative splicing events were identified in this study, which suggests a limited impact of these processes in diversification of the *P. falciparum* gene pool.

In a subsequent study, RNA-Seq data from four time points of the *P. falciparum* IDC were interrogated in greater depth, which led to discovering 977 previously undetected splicing sites and an additional 310 alternative splicing events in 254 genes (Sorber 2011). Interestingly, only one third of these splice variants were in-frame transcripts (expected by chance), which suggests that alternative splicing might not play a major role in diversification of protein sequences but rather regulates gene expression via the nonsense-mediated decay mechanism (NMD). The discovery of multiple complex splice arrangements made in this study, including splice variants from two opposing strands (antisense splicing), may support this model (see below).

The periodic and highly regulated mode of transcription during the *P. falciparum* IDC appears to be highly conserved between different *P. falciparum* parasite populations. Reconstructing the IDC transcriptional cascade for several *P. falciparum* laboratory strains (Llinas 2006) as well as freshly adapted field isolates (Mackinnon 2009) showed that the vast majority of genes have identical temporal expression patterns. Only about 1.3% (69) of *P. falciparum* genes showed transcriptional shift greater than 12 hours between three studied laboratory strains (HB3, 3D7 and Dd2), most of which belong to genes involved in host–parasite interaction, invasion, and antigenic variation (Llinas 2006). Molecular mechanisms underlying such transcriptional shifts are currently not known. Besides these, the vast majority of the IDC cascade is highly conserved, suggesting its importance in parasite adaptation to its host environment.

In contrast to *P. falciparum* strains, the IDC transcription pattern is not fully identical among *Plasmodium* species. Only about 68% syntenic genes between *P. falciparum* and *P. vivax* exhibit the same expression timing, and 22% and 10% of genes exhibit moderate and severe alterations in their temporal transcription pattern, respectively (Bozdech 2008). These observations originated from studies in which microarrays were used to establish the IDC transcriptome of *ex vivo P. vivax* parasite cultures. At least 3500 (~70%) were found to be expressed and stage-specifically regulated during the *P. vivax* IDC in a similar fashion to *P. falciparum*.

However, in *P. vivax*, several crucial biological processes appeared to be targeted to a different developmental stage of the IDC. In particular, factors of host cytoplasm remodeling and hemoglobin degradation appear to be expressed later in schizont stages in *P. vivax* compared to the ring stage in *P. falciparum*. This suggests that *P. vivax* has rearranged the sequence of biological processes associated with the progression of its IDC, which may reflect its species-specific adaptation to its host environment. Even for biological pathways that are conserved in terms of the timing of their gene expression contain at least one or a few genes whose expression was shifted into a different IDC stage in *P. vivax* compared to *P. falciparum* (Bozdech 2008).

In future studies it will be interesting to investigate the role of these genes in the speciation of the *Plasmodium* genus either as rate-limiting factors or as developmental gate-keepers of essential biological processes. With this goal, more studies are currently ongoing to investigate the conservation of gene expression in the IDC across a large panel of *Plasmodium* species (Preiser and Bozdech, unpublished data).

Sexual development and exoerythrocytic stages

Similarly to the asexual blood stages, tight transcriptional regulation was demonstrated for the sexual development as well as other exoerythrocytic forms. The initial microarray-based analysis of the *P. falciparum* life cycle uncovered a specific set of 152 and 218 genes with enriched or exclusive expression, respectively, in gametocytes. Interestingly, 80% of these genes were classified as hypothetical proteins, which signals their unique sequence and thus involvement in unique *Plasmodium* functions (Le Roch 2003).

A subsequent time course analysis expanded on this initial finding exploring global transcriptional profiles across the entire gametocytogenesis from stage I to stage V (Young 2005). Overall, 3410 genes were found to be involved in gametocytogenesis by being expressed at least at one time point of the experimental time course. This approach brought more insights into the possible function of previously known factors of sexual development by classifying these based on their peak expression. While some of these, such as *pfg27/25*, *pfs16*, *alpha tubulin ii*, and *pfs48/45*, seem to be important for the early gametocytogenesis being expressed during stage I through III, others, such as *pfs25*, *pfs28*, *pf77*, and *dmc1-like protein* are essential for the final stages of sexual development being expressed during stages IV and V.

In addition to the known factors, this dataset allowed identification of new gametocyte-linked genes. Using a specific technique developed for bioinformatics analyses of *Plasmodium* gene expression data called ontology-based pattern identification (OPI), the authors of this study could classify an additional 246 genes as sexual-development specific. Of these, 76% represent unique *Plasmodium* sequences. This further underscores the presence of evolutionarily diverse processes in the *Plasmodium* sexual stages.

Interestingly, exploration of the promoter regions of the 246 genes identified an overrepresentation of a palindromic sequence TGTANT-NTACA which could serve as a binding site for a putative transcription factor. Although this motif has not been verified experimentally, its presence is essential for expression of a gametocyte gene, *pfs25* (Dechering 1999). This time-course experiment brought many insights into the sexual development, but very little is known about the commitment to gametocytogenesis that takes place during the preceding round of an asexual cycle.

To address some of these issues, Silvestrini and colleagues (Silvestrini 2005) have carried out comparative transcriptomics studies between a gametocytogenesis defective strain (F12) and its parental 3D7 (with an intact potential for sexual development). According to the expectations, two major gametocyte factors, *pfg27/25* and *pfs16*, were suppressed in F12 compared to 3D7. Using these two transcription profiles as templates, a gap statistical approach has identified an additional 122 genes suppressed in the F12 during the presumed time of commitment and early stages of gametocytogenesis. From these, only 32% represented genes with function annotations, whereas the rest were (once again) *Plasmodium* specific. Interestingly, two factors of the cyclic AMP signaling pathway, a putative cAMP 3′5′-cyclic phosphodiesterase and a diacylglycerol kinase, were also found to be suppressed in F12, which is in concordance with previous suggestions of the role of cAMP signaling pathway in commitment (Inselburg 1983). Finally, two novel proteins, PFL0792c and PF10_0164, were identified as new factors involved in the early gametocyte (stage I and II) formation and thus named *P. falciparum* proteins of early gametocytes *pfpeg3* and *pfpeg4* (Lanfrancotti 2007).

In spite of these achievements, many questions about transcriptional regulation during the sexual stage development remain unanswered. Given the complex transcriptional pattern uncovered by these early studies, it will be interesting to uncover the complete set of *cis* and *trans* regulatory factors involved in both commitment and gametocyte maturations. For that, more transcriptomics studies are likely to emerge in the near future.

Similar to the sexual development, the in-mosquito and subsequent hepatocytic developmental stages (Tarun 2008) have received a substantial attention by transcriptomics studies. First, a comparative transcriptomics analysis was carried out between oocyst-derived sporozoites (ooSpz) of *P. yoelii* collected 10 days after infection and salivary gland–derived sporozoites collected 5 days later (Mikolajczak 2008). A total of 124 and 47 unique genes were found to be specifically upregulated in sgSpz and ooSPZ, respectively. Of these, 31 sgSpz- and 15 ooSpz-specific genes were found to encode proteins that carry a signal peptide and/or transmembrane domain and thus might be involved in hepatocyte and salivary gland invasion, respectively. One of these genes, termed *UIS3* (PY04986), was followed up for functional analysis, and it was determined that targeted disruption of *UIS3* results in the failure of the parasites to invade salivary glands. UIS3 protein was found to be localized in the apical compartments of both ooSpzs and sgSpzs, which suggest its direct role in the invasion process.

Transcriptional regulation

Basal transcriptional machinery and transcription factors

Plasmodium has overall similar mechanisms of gene regulation, like other eukaryotes, with general transcription factors recruiting RNA polymerase II to promoter regions (Coulson 2004; Callebaut 2005; Bischoff and Vaquero 2010). Transcription is shown to be monocistronic (Lanzer 1994), and protein-encoding genes are transcribed by RNA polymerase II (Lanzer 1992). Although about 60% of the genome is transcriptionally active, very few specific transcription factors have been identified to date in spite of various computational and molecular experimental efforts. Lack of specific transcription factors suggests that the transcriptional cascade is regulated by combinatorial mechanisms involving interactions of transcription factors with other regulatory elements like epigenetic marks. Different studies have led to the identification of various regulatory sequence elements controlling gene expression for some of the genes (Lanzer 1992; Horrocks and Lanzer 1999; Osta 2002; Militello 2004; Tham 2007).

The best-studied transcription factors in *Plasmodium* belong to the apicomplexan Apetala2 (ApiAP2) class of transcription factors that are homologous to plant AP2 transcription factors predominantly involved in developmental processes and stress responses (Balaji 2005). The ApiAP2s characterized by one or more DNA-binding motifs are conserved among apicomplexans (Balaji 2005; Iyer 2008), with *P. falciparum* having 27 members of this family (Balaji 2005; Campbell 2010). Figure 8.1 shows the AP2 domains found in 20 ApiAP2 proteins along with their transcriptional profiles and reveals that these transcription factors are expressed throughout the entire developmental cycle.

Several functional genomics efforts have now attempted to assess the role of ApiAP2 in the IDC transcriptional cascade. Using a protein-binding microarray (PBM) containing all possible 10-mer DNA sequences spread across 44,000 double-stranded DNA 60-mer oligonucleotides, it was shown that two members of the ApiAP2 family (PF14_0633 and PFF0200c) bind to palindromic motifs similar to binding sites of other eukaryotic transcription factors (De Silva 2008). The binding-site motifs identified for these two ApiAP2 are located in multiple promoter regions of *P. falciparum* genes. Interestingly, the average expression profiles of genes containing the putative PFF0200c

Domain ID	Gene ID_PlasmoDB Version 9	Motif	Expression profile
PFE0840c_D2	PF3D7_0516800	GACATC	
PFF0670w_D1	PF3D7_0613800	TAAGCC	
PFF0670w_D2	PF3D7_0613800	CTCTAG	
PFF0200c_DLD	PF3D7_0604100	GTGCAC	
MAL8P1.153	PF3D7_0802100	ACACAC	
PF11_0091	PF3D7_1107800	GCATAC	
PFD0985w_D1	PF3D7_0420300	GTGTGT	
PFD0985w_D2	PF3D7_0420300	TGTTAC	
PF11_0442	PF3D7_1143100	GCTAGC	
PF10_0075_D1	PF3D7_1007700	GTCGAC	
PF10_0075_D2	PF3D7_1007700	TCTTGC	
PF10_0075_D3	PF3D7_1007700	GTGCAC	
PFL1075w	PF3D7_1222400	TATATA	
PFL1900w_DLD	PF3D7_1239200	TCTACA	
PF14_0533	PF3D7_1456000	CACACA	
PF13_0097	PF3D7_1317200	AGCTCA	
PF11_0404_D1	PF3D7_1139300	AGAACA	
PF13_0235_D1	PF3D7_1342900	GCCCCG	
PF13_0267	PF3D7_1350900	CTAGAA	
PF07_0126_DLD	PF3D7_0730300	ATTTTCC	
PF14_0079	PF3D7_1408200	GCAACC	
PF14_0633	PF3D7_1466400	GCATGC	

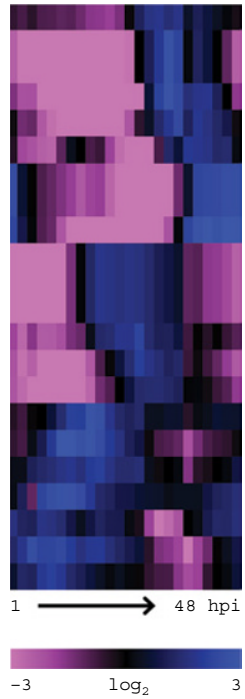

Figure 8.1 Overview of transcription profiles of the ApiAp2 genes. The figure shows a list of AP2 domains of 20 ApiAP2 proteins, which are predicted to bind with DNA motifs in *P. falciparum* (Campbell 2010). The name of the AP2 domains, corresponding PlasmoDB gene ID, primary motif recognized, and IDC transcriptional profile are listed in the first, second, third, and fourth columns, respectively. D1, D2, and D3 refer to the domain number on the protein, and DLD refers to two domains (domain linker domain). The transcriptional profiles shown are taken from a previous study (Foth 2011), where microarrays were carried out at 24 time points across the 48-hour IDC. (*See insert for color representation of this figure.*)

binding site correlate well with the transcription profile of PFF0200c itself, thereby further strengthening the original hypothesis that PfAP2s play a role in transcriptional regulation of the IDC. The identification of binding sites of other ApiAP2s (Campbell 2010) revealed that a number of ApiAP2 proteins bind multiple, distinct motifs, which could be attributed to complex transcriptional regulatory networks governed by the ApiAP2 family.

Among the other transcription factors, PfMyb1, the homologue of Myb eukaryotic transcription factor was shown to alter the mRNA levels of a considerable number of genes (Gissot 2005). A comprehensive directory of transcription-associated proteins (TAPs) has been created using *in silico* reports and databanks (Bischoff and Vaquero 2010), where around 200 TAPs are clustered as general transcription factors, chromatin-related proteins, and specific transcription factors. It will be interesting to functionally approve the role of these predicted factors of transcriptional regulation.

Promoters

Promoter sequence and activity has a major impact on gene regulation. Unfortunately, the sequence organization of *Plasmodium* (AT richness) has limited our knowledge of promoter-specific regulation. Various attempts have been made to identify core promoters and transcriptional start sites

(TSS) (Brick 2008; Ponts 2011), many of which are still under validation. However, *P. falciparum* promoters have the characteristic bipartite structure of eukaryotic promoters, consisting of a basal promoter regulated by upstream enhancer elements (Crabb and Cowman, 1996). Promoters also contain motifs for binding sites of known common transcription factors, although the functionality of these motifs in *Plasmodium* has not yet been demonstrated (Lanzer 1992; Lanzer 1993).

Transcriptional analyses and transfection experiments revealed the presence of constitutive promoters expressed throughout the life cycle (Crabb and Cowman, 1996), as well as stage-specific promoters that show stage-specific activity (de Koning-Ward 1999; Dechering 1999; Wickham 2003). Although *Plasmodium* does not recognize other eukaryotic promoters, promoters from different species of *Plasmodium* are functional in heterologous transfection experiments, indicating a conserved mechanism of transcriptional regulation (Crabb and Cowman 1996; Fernandez-Becerra 2003; Mota 2001; van der Wel 1997). However, *P. vivax* promoters were found to be poorly recognized by *P. falciparum* machinery (Azevedo and del Portillo 2007), suggesting that the presence of species-specific recognition and regulation are important as well.

Epigenetic mechanisms

Epigenetic mechanisms have been implicated as one of the key players in the dynamic gene-regulation pattern in *P. falciparum*. The epigenetic machinery in *Plasmodium* consists of factors that are conserved among the eukaryotes as well as some unique features. Unlike the other eukaryotes, the *Plasmodium* epigenome is mainly eukaryotic with few well-defined heterochromatic islands (Lopez-Rubio 2009; Salcedo-Amaya 2009). The linker histone H1 is lacking (Miao 2006), and histone variants are reported to carry unique set of acetylations (Trelle 2009). Absence of DNA methylation (Choi 2006) and a functional RNA interference system (Baum 2009) suggest the involvement of alternative regulators of epigenetic processes in *P. falciparum*. The post-translational modifications on histone tails of *P. falciparum* have emerged as crucial regulators in gene-expression patterns. Using chromatin immunoprecipitation coupled with microarray (ChIP-on-chip) as well as immunoprecipitated chromatin subjected mass parallel sequencing (ChIP-Seq), genome-wide distribution of a few modifications has been described for *P. falciparum* but until recently, their role in transcription remained unknown.

The *var* family of clonally variant genes presented one of the first evidences of epigenetic regulation in *P. falciparum* (Voss 2003). Methylation of lysine 9 on histone H3 (H3K9me3) in *P. falciparum* is associated with clonally variant gene families clustered mainly on subtelomeric regions, with some chromosomal internal regions (Lopez-Rubio 2009). The replacement of H3K9me3 at 5′ flanking regions of *var* genes by acetylation (H3K9ac) led to *var* gene activation (Chookajorn 2007; Lopez-Rubio 2007). Furthermore, studies of clonally variant expression control at the epigenetic level revealed transcriptional heterogeneity among isogenic parasite lines (Rovira-Graells 2012). The functionally unrelated variantly expressed gene families in these isogenic parasite lines were regulated by epigenetic marker H3K9me3. It has been demonstrated that antigenic switching of *var* genes implies epigenetic memory, among other factors (Fastman 2012; Merrick 2012).

The euchromatic markers H3K4me3 and H3K9ac were shown to be spread evenly upstream and downstream of active and inactive genes in rings but associated with the 5′ end of active genes in schizonts (Flueck 2009). Subsequent ChIP-Seq studies showed that both of these histone modifications associated mainly with promoter regions, but only H3K9ac is correlated with transcription, whereas H3K4me3 is uncoupled from transcription (Bartfai 2010). Both these marks were shown to colocalize with the histone variant H2A.Z at upstream intergenic regions (Bartfai 2010). H2A.Z-containing nucleosomes stably demarcate intergenic regions of the *P. falciparum* genome from coding regions that are occupied by H2A. However, H2A.Z occupancy at promoter regions of euchromatic genes does not correlate with transcription levels. In contrast, occupancy of H2A.Z at *var* gene

promoters strongly associates with transcriptional activity (Petter 2011). Overall, enrichment at active euchromatin as well as heterochromatin regions shows the importance of H2A.Z in transcriptional activation.

A comprehensive epigenomic map was generated in order to map the temporal pattern of histone H3 and H4 modifications with transcriptional activity along the genome (Gupta 2013). Similar to mRNA, the occupancy patterns of dynamic histone modifications exhibited single peak profiles, with each locus being marked once at a specific time during the IDC. The comparative analysis between histone marks and mRNA levels allowed a direct association between occupancies of the individual histone modification and mRNA levels genome-wide across the IDC. This study of 13 histone marks using ChIP-on-chip at 6 time points across IDC distinguished between stable marks (H4K5ac, H3K14ac, H4R3me2, and H3K79me3), which occupy the genome almost throughout the IDC, and dynamic marks (H4K8ac, H4K12ac, H4K16ac, H4-tetra-ac, H3K56ac, H3K9ac, H3K4me3, H4K20me1, and H4K20me3), which show dynamic changes in their occupancy across the IDC. Although most of the histone marks showed maximum occupancy at intergenic and promoter regions, the dynamic occupancy changes were reflected at coding regions, suggesting that the intergenic regions with marked histones may function as general demarcation elements, but it is mainly the coding regions that play a role in dynamic transcriptional activity. Importantly, eight modifications displaying tight correlations with transcript levels showed differential affinity to distinct genomic regions, with H4K8ac occupying predominantly promoter regions, while others occurred at the 5′ ends of coding sequences.

Overall, these observations reveal that gene regulation is responsive to dynamic pattern of specific histone marks in *P. falciparum* life cycle. The unique plasticity of the distribution of epigenetic marks during *Plasmodium* development is not seen in other eukaryotes. This indicates the existence of unique molecular mechanisms controlling chromatin remodeling, as well as mechanisms that link epigenetic markers with transcription. Targeting of these molecular mechanisms has a high potential for development of new malaria-intervention strategies.

DNA-binding proteins involved in chromatin remodeling also play a major role in regulation of gene expression. *P. falciparum* heterochromatin protein-1 (PfHP1), a homologue of highly conserved protein implicated in heterochromatin formation in most eukaryotes (Grewal and Jia 2007), was shown to specifically interact with H3K9me3 (Perez-Toledo 2009). High resolution ChIP-on-chip analyses revealed an exclusive association of PfHP1 with all previously identified heterochromatin regions of the *P. falciparum* genome, including the TARE1-6, and the VSA gene families (Flueck 2009). PfHP1 and H3K9me3 enrichment correlated throughout the genome, confirming the involvement of PfHP1 in heterochromatin formation and silencing of *var* and other gene families involved in host–parasite interactions.

Unlike in other eukaryotes, the centromeres in *P. falciparum* are devoid of HP1-associated pericentric heterochromatin and are co-occupied with PfCenH3 and PfH2A.Z (Hoeijmakers 2012). This suggests a unique role of epigenetic environment in centromere establishment, which may be important for efficient chromosomal segregation. PfHP1 is also associated with another heterochromatic marker, PfSIP2, a member of the ApiAP2 family of transcription factors (Balaji 2005; De Silva 2008; Flueck 2010). PfSIP2 associated exclusively with the telomeric ends of *P. falciparum* chromosomes, recognizing specific SPE2 DNA motifs that are found in a subset of subtelomeric *var* promoters and in telomere-associated repeat regions 2 and 3 (TARE 2-3) (Flueck 2009). Although overexpression of PfSIP2 in *P. falciparum* cells had no effect on gene expression, this protein is essential for the silencing of a subset of *var* promoters and hence maintenance of subtelomeric heterochromatin. Apart from these heterochromatic markers, Volz and colleagues identified at least 24 novel *Plasmodium* nuclear proteins, many of which could be linked to chromatin remodeling (Flueck 2009). Their role in transcriptional regulation needs to be investigated.

Non-coding RNAs

In eukaryotes, noncoding RNAs (ncRNAs) form a part of epigenetic regulatory system and control many nuclear processes. Long noncoding RNAs (lncRNAs) and small noncoding RNAs interact with RNA binding proteins or chromatin remodeling complexes. In *P. falciparum*, ncRNAs are shown to be associated with centromeric chromatin (Li 2008) as well as telomeric and subtelomeric regions (Voss 2003). Raabe and colleagues identified 630 nonprotein coding RNA candidates with implication of these in regulating housekeeping genes and virulence factors (Raabe 2010). Using high-resolution DNA tiling microarray, 60 putative *P. falciparum* lncRNAs were found that are under developmental regulation during the parasite's pathogenic human blood stage (Broadbent 2011). Out of these 60, 22 were grouped as telomere-associated repetitive element transcripts (lncRNA-TARE). The lncRNA-TAREs exclusively mapped to the TARE regions on the ends of the chromosomes and are implicated in the mechanism of *var* gene regulation.

Transcriptional variation

Variatome

Until recently, most transcriptional studies focused on transcriptional changes in a population of parasites. Work using parasites recloned from a single parasite clone indicated a significant heterogeneity of transcription within genetically homogeneous parasites. This transcriptional heterogeneity mainly involved genes important in parasite–host interactions and is thought to represent a bet-hedging strategy that provides the parasite with the ability to rapidly adapt to changes in the host environment.

Clonally variant gene expression where two parasites with identical genomes establish differential expression of the same gene has been described for many gene families including *var* genes, genes linked to erythrocyte invasion, and exported proteins (Cortes 2007; Lavazec 2007; Scherf 2008). A comprehensive study of 21 parasite lines was done to characterize the genome-wide extent of clonal variation (Rovira-Graells 2012). The transcriptional variability in the genetically identical subclones was not restricted to antigenic variation but also affected genes linked to lipid metabolism, protein folding and erythrocyte remodeling. Interestingly, the diverse clonally variant gene families were linked to a common epigenetic mechanism of H3K9me3 -based heterochromatin formation (Flueck 2009; Lopez-Rubio 2009; Rovira-Graells 2012). Clearly, chromatin changes linked to clonally variant gene expression brings out another important aspect of epigenetic inheritance and will be important in future to reveal mechanisms underlying variant expression.

In vivo versus *in vitro*: Comparison of transcription profiles

A significant limitation of most of the transcriptional studies to date is the fact that the data are generally obtained from parasite isolates that have been maintained in culture for years, if not decades. This therefore raises a valid concern about whether the transcriptional profiles obtained, particularly in relation to genes involved in host–pathogen interactions, are still meaningful. There are, however, a number of challenges in obtaining transcriptomic data from clinical isolates. These include getting well-staged parasites as well as obtaining sufficient amount of RNA. In addition, in *P. falciparum*, only ring-stage parasites are normally obtained from the patient, requiring at least some short-term culture if the later stages are to be studied.

As a first step to overcome some of these challenges it is feasible to culture adapt new parasite isolates for subsequent transcriptomic analysis. These *in vitro* cultures generate sufficient material to allow whole microarray as well as RNA-Seq analysis. Comparison of the IDC transcriptomes of six

freshly culture-adapted parasite isolates from Kenya with three long-term *P. falciparum* laboratory strains (Mackinnon 2009) identified a number of striking differences. The differentially expressed genes confer important functional groups like host–parasite interactions, sexual differentiation, transcription and translation, protein folding, and biosynthesis. The majority of these genes were upregulated in the field isolates, indicating a loss of function during long-term culture adaptation. This made it clear that to fully understand host–parasite interactions and obtain a complete understanding of unique parasite biology and virulence factors linked to disease, fresh clinical isolates need to be analyzed.

With this in mind, transcriptomic studies of field isolates and *in vivo* transcriptome under host response were initiated. Relatively low amount of parasite sample has always been a drawback in transcriptional profiling of field samples. Nevertheless, initial studies analyzed the genome-wide gene expression profiles of field samples from Senegal and Nigeria, which were compared to ring-stage transcriptome of *in vitro* cultures (Daily 2004; Daily 2007). Major transcriptional differences for many metabolic enzymes and basic cellular processes were observed, suggesting the existence of distinct physiological states of the *P. falciparum* parasites in infected patients (Daily 2007). Another study showed overexpression of gene families that encode surface proteins in field isolates (Daily 2005). This was subsequently confirmed at the protein level for the STEVOR variant antigen family, where it was shown that clinical isolates had significantly higher levels of expression than observed in long-term culture-adapted parasites (Blythe 2009). The field isolates showed increased expression levels of several other genes, including RESA-2 (PF11_0512), putative long-chain fatty acid ligase (PFC0050c), and one member of a hypothetical protein family (PF14_0752), which was later annotated as the *Plasmodium* helical interspersed subtelomeric family (PHIST) (Sargeant 2006).

All parasites obtained from pregnancy-associated malaria (PAM) exhibit uniform cytoadherance independent of their geographical origin (Fried 1998). Hence, identification of genes expressed in PAM may provide important targets for vaccine development. To study PAM, direct transcription profiling of infected placental samples has been done. Five genes with Pexel transport motif and one *var2csa* gene were highly expressed in whole-genome expression analysis carried out from placental parasites from pregnant women (Francis 2007). These findings suggest that a group of genes may be important for binding phenotype of *P. falciparum* in the placenta. Another similar study conducted on 18 infected placental samples from patients in Senegal showed significant enrichment of subtelomeric genes as well as genes encoding exported proteins (Tuikue Ndam 2008). One of these proteins, PFI1175w, was exclusively expressed in placenta-derived *P. falciparum,* with no expression being detected in parasites from peripheral blood of pregnant women or in *in vitro*–selected CSA-binding laboratory lines (Tuikue Ndam 2008). PFI1175w is a member of the subtelomeric PHIST gene family, whose other member, PF14_0752, showed strong overexpression in the earlier field isolate studies (Daily 2005).

Transcriptional variation was also observed in *P. vivax* field isolates. The transcriptomes of three isolates from northern Thailand showed significant variation in transcript levels of genes associated with invasion, reticulocyte-binding proteins, antigenic variation, host–parasite interactions, and also several metabolic functions like glycolysis (Bozdech 2008). *In vivo* profiling of eight *P. vivax* isolates collected in Peru (Westenberger 2010) showed that the first three enzymes of the glycolytic pathway were expressed at similar levels between isolates, whereas the eight downstream proteins in the pathway exhibited up to 100-fold difference in expression between individual isolates.

Overall, *in vivo* whole-genome analysis revealed a modulated expression pattern for genes involved in host–parasite interactions, energy metabolism, stress response, and sexual development, opening avenues for identification of new intervention targets.

Transcriptional changes in response to outside stimuli

A number of studies investigating the transcriptional response to antimalarial drugs indicated a minimal transcriptional response by the parasite, leading to the proposition that *Plasmodium* transcriptional regulation is hard-wired. This was supported by the initial analyses of the effect of the antimalarial drug chloroquine on *P. falciparum,* which could not identify any specific high-amplitude changes in mRNA abundance (Gunasekera 2007), even when cells were treated with a 400-nM concentration for 6 hours. Similar low transcriptional responses to antifolate drugs (Ganesan 2008), or calcium-dependent protein kinase (PfCDPK1) inhibitor purfalcamine (Kato 2008), also suggested hard-wired metabolic transcriptome.

However, the bisthiazolium compound T4, a choline analogue with a strong potency against malaria parasites, elicited dramatic transcriptional changes, with 137 genes showing more than two-fold differential expression after 24 hours (Le Roch 2008). The transcriptional changes involved genes of sexual development, fatty acid biosynthesis in the apicoplast, microtubule cytoskeleton, and several generic cellular pathways including glycolysis, protein metabolism, RNA processing, cell cycle and DNA replication. Similarly, a lethal dose of artesunate, one of the important derivate of artemisinin, induced dramatic transcriptional responses involving 398 (8.5%) genes with 244 overexpressed and 154 underexpressed (Natalang 2008). Differentially expressed genes belonged to chaperones, transporters, cell cycle regulation, zinc-finger proteins, transcription activity, and redox metabolism. Some other growth perturbation studies reported the differential expression of genes linked with various biological processes that may be direct or indirect transcriptional responses (Oakley 2007; van Brummelen 2009; Torrentino-Madamet 2010).

These approaches have also been useful in the study of epigenetic regulators by analyses of the effect of specific inhibitors of chromatin-remodeling enzymes on *Plasmodium* transcription. These include the histone deacetylase (HDAC) inhibitor apicidin (Chaal 2010) and the histone acetylase (HAT) inhibitor anacardic acid (Barski 2007; Cui 2008), both of which can induce specific transcriptional changes linked to changes in euchromatin markers. Apicidin caused profound transcriptional changes in multiple stages of the *P. falciparum* IDC, including the induction of genes suppressed under normal growth conditions of that particular stage of the parasite (Chaal 2010). Inhibition of HDAC activity led to genome-wide hyperacetylation, leading to deregulation of the IDC transcriptional cascade. It was also shown that HDAC inhibitor apicidin affected transcription of a number of ApiAP2 genes (Chaal 2010), suggesting the association of these transcription factors with epigenetic machinery. In our more-recent study, we showed that transcriptional changes observed after 2 hours of exposure to three different HDAC inhibitors were transitory, with only 1% to 5% of genes being regulated after removing the compounds (Andrews 2012). Overall, these data clearly demonstrate that the transcriptome is not hard-wired but is able to rapidly respond to stimuli from the environment.

Growth perturbations as a tool for gene annotation

Nearly 50% of the predicted genes in the genome of *P. falciparum* have no known function, and new approaches are needed to facilitate the assignment of function. The functional analyses of transcriptional responses to growth perturbations is useful for annotation of novel uncharacterized genes and for understanding the mode of action of chemotherapeutic agents and other compounds in drug development (Hughes 2000).

With this in mind, an extensive transcriptional profiling was done using 20 chemical perturbations using a microarray dataset consisting of 23 time points across the life cycle of *P. falciparum* (Hu 2010). Whereas some perturbations, such as inhibitors of proteases or microtubule formation, induce minimal mRNA changes affecting less than 1% of the genome, other compounds, such as specific inhibitors of calcium- and calmodulin-dependent kinases and calcineurin pathway, caused

specific high-amplitude transcriptional responses (>2-fold) that involved 5% to 10% of *Plasmodium* genes. In addition, some inhibitors including staurosporine, (PKs inhibitor), apicidin and trichostatin A (HDAC inhibitors), and EDTA (chelator of ambient Ca^{2+}) caused wide-range transcriptional changes that were consistent with developmental arrests. Importantly, transcriptional changes induced by growth perturbations are highly consistent with the functional assignment of the affected genes, which indicates common transcriptional regulation for functionally related genes (Hu 2010). Overall, this strategy has made it possible to functionally characterize a large number of unknown genes in *P. falciparum.*

Transcription and drug resistance

Artemisinin resistance in *P. falciparum* malaria has alerted scientists to look into the mode of action of resistance or delayed clearance phenotype. To identify key features associated with the delayed parasite clearance phenotype, transcriptional profiling of parasites collected from patients in Pailin, Western Cambodia was performed (Claessens 2011). Blood samples were collected from patients with both fast- and slow-clearing malaria infection after artemisinin treatment, and parasites were cultured for another 48 hours *in vitro.* The transcriptomes generated across the life cycle (6-hour resolution interval) were compared to transcriptomes of six other normal clearance isolates from Southeast Asia generated in a similar fashion. Parasites with slow clearance rates showed reduced expression of many cellular and metabolic pathways during early stages of the IDC, whereas in the later stages, there was an increased expression of genes associated with protein metabolism. The study has provided a large amount of information regarding candidate genes and markers associated with artemisinin resistance.

Biological insights

The ability to extensively study the transcriptional status of the parasite at any given point during its development now provides an opportunity to correlate changes in transcriptional patterns to the parasite's phenotype or behavior. Moreover, it now also allows the study of clinical isolates and correlate transcription to clinically relevant criteria.

Proteomics

There is mounting evidence that *Plasmodium* gene regulation is mediated not only by transcriptional factors but also by post-transcriptional, translational, and post-translational factors. Functional analysis of *Plasmodium* proteins has shown their involvement and important role in many processes. Initial proteome studies showed that asexual blood stages expressed a large number of proteins involved in *Plasmodium*-specific functions along with other basic requirements in a cell (Florens 2002; Lasonder 2002). Out of 2400 proteins profiled, 839 and 1036 distinct proteins were identified in the merozoite and trophozoite stages, respectively (Florens 2002). The study also identified some chromosomal clusters encoding for coexpressed proteins, hinting at a possible mechanism of regulation. In another study, Lasonder and colleagues identified 1289 proteins out of which 714 proteins were detected in the asexual blood stages, 931 in gametocytes, and 645 in gametes (Lasonder 2002). Most of the asexual-stage proteins were assigned to host–parasite interaction processes. Similarly, *P. berghei* proteomics data identified 1836 stage-specific proteins (Hall 2005). Interestingly, more than half of the proteins were detected in only one stage of the life cycle. These studies proved the timely expression of proteins at every stage.

Nirmalan's group used metabolic labeling of heavy isotope–containing isoleucine during normal growth followed by two-dimensional gel electrophoresis and mass spectrometry (Nirmalan 2004). They show that during the IDC, there is delay between peak mRNA and protein abundance. Daily and colleagues compared mRNA and protein levels across seven major developmental stages of the *P. falciparum* life cycle (Daily 2004). They detected a total of 2584 proteins across seven stages. Similar to previous observations, they also found that although correlated in their profiles, a significant fraction of genes exhibited a delay between the peak abundance of mRNA and protein.

Foth and coworkers carried out quantitative two-dimensional differential gel electrophoresis (2D-DIGE) followed by tandem mass spectrometry (MS/MS) between 34 and 46 hpi and showed differential abundance of 278 proteins (Foth 2008). They show examples of correlations with lag between mRNA and protein peaks as well as anti-correlations between transcript and protein profiles. This study was further extended across the 48-hour IDC with microarray and 2D-DIGE carried out at every 2 hours (Foth 2011). A total of 1183 protein/isoforms were found to change their abundance, with the majority of proteins peaking in either early ring stage or mid-schizont stage. The 125 genes identified by MALDI-TOF/TOF represented several major functional groups. Again, a majority of the proteins exhibited 6- to 16-hour delay in their peak abundance compared to their transcripts. Overall, these results depict a tight regulation between transcriptome and proteome, with transcripts appearing earlier than proteins.

Importantly, similar to transcript abundance or many histone modifications occupancy, most proteins also exhibit cyclic abundance profiles with one peak during the IDC (Figure 8.2). Such a broad and dynamic character of regulation is unprecedented among known living organisms and likely represents a unique evolutionary adaptation of the parasite to its host.

Translational regulation

Post-translational modifications

Many proteins in *Plasmodium* occur in multiple isoforms, which may be due to post-translational modifications (PTMs) or other pretranslational events. Apart from the PTMs occurring on histones (Salcedo-Amaya 2009), which are discussed in earlier sections, other proteins in *Plasmodium* also display translational regulation *via* PTMs. From the small-scale proteome studies, 10 out of the 28 (Nirmalan 2004) and 24 out of the 54 (Foth 2008) proteins identified were found to be present in two or more isoforms. The expression patterns of these isoforms of proteins varied dramatically from each other in many instances, revealing the importance of such PTMs in expression patterns. From the large-scale proteome analysis, 125 *P. falciparum* proteins were represented by a total of 363 protein spots on the 2D-DIGE gels (Foth 2011), which suggests the presence of extensive PTMs in the *Plasmodium* proteome during the IDC.

Proteolytic processing, one of the major modes of post-translational control, has been demonstrated to play a major role in many crucial *Plasmodium* functions, including hemoglobin degradation, merozoite egress, and invasion (Chung 2009). In studies by Foth and colleagues, around 46 proteins were found to be truncated, and several proteins, including falcipains 2A and 2B and plasmepsins I, II, III (HAP), and IV, were detected at lower-than-expected molecular weights (Foth 2011). The other major PTMs identified in *P. falciparum* are phosphorylation, acetylation, methylation, ubiquitination, glycosylation, and lipidation (Chung 2009). It was shown that around 170 proteins are phosphorylated at a serine or tyrosine residue (Wu 2009). Out of these, some phosphorylated proteins showed constitutive expression, whereas others displayed stage-specific expression. In all, such processes of modifications diversify the proteome of *Plasmodium*, which may be important for stage-specific expression or cellular targeting.

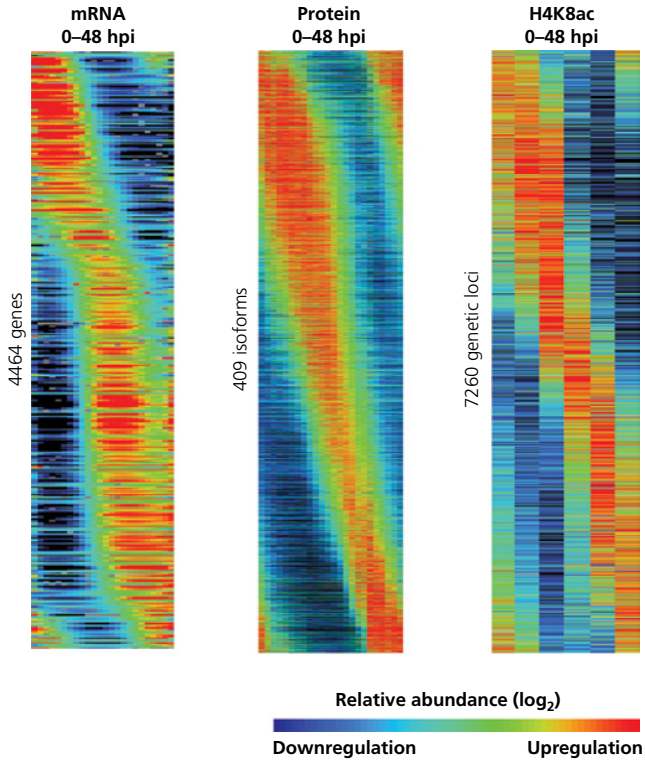

Figure 8.2 Overview of the expression, protein, and histone modification profiles during *P. falciparum* IDC. The figure depicts abundance profiles of 4464 transcripts (corresponding to 4464 genes), 409 protein isoforms (corresponding to 149 genes), and 7260 acetylated histone H4 lysine 8 (H4K8ac) profiles (corresponding to 3108 genes) across the IDC in *P. falciparum*. The results are based on oligonucleotide microarray for mRNA or histone-modification profiles and 2D-DIGE for protein profiles and show the prevalence of single peak profiles across the IDC in each case. The heat maps depicting mRNA (on the left), protein (in the middle) and H4K8ac (on the right) abundance profiles comprise 24, 24, and 6 time points, respectively, covering 0 to 48 hours after invasion (hpi). Note that the rows in each heat map representing transcripts, proteins, and H4K8ac *do not* correspond to one another. Each row represents the transcript-abundance profile for each gene, protein-abundance profile for each protein isoform, and H4K8ac-abundance profile for each genetic locus as seen in left, middle, and right panels, respectively. The abundance profiles were sorted according to their Fourier phase, and the color scale represents the lowest smoothed profiles calculated from centered curves of relative $\log_2$ occupancy ratios. Data for this figure were derived from 2 previous studies (Gupta 2013; Foth 2011). (*See insert for color representation of this figure*.)

Organellar translation

The complex biology behind protein architecture has led to several subproteomic studies targeting individual compartments of the parasite cell. Apart from cytosol, apicoplast and mitochondria are the other two translationally active sites in *Plasmodium* (Chaubey 2005; Feagin 1992), and most of the nuclear encoded factors required for translation are imported into these organelles. The cytosolic, apicoplast, and mitochondrion protein-translation machineries are responsible for translation of approximately 5000 nuclear-encoded genes, 50 apicoplast-encoded genes and 3 mitochondrial-encoded genes, respectively (Feagin 1992; Gardner 2002). Only two genome compartments out of three – namely nucleus and apicoplast – encode tRNAs in *Plasmodium*. Whereas other eukaryotes have around 60 amino acid tRNA synthetases, *Plasmodium* has only 37, out of which 23 have signal

peptides targeting them to different subcellular locations (Bhatt 2009). This suggests the role of tRNA synthetases at more than one location in order to complement the missing counterparts.

Plasmodium possesses cytoplasmic rRNAs as well as mitochondrial and plastid rRNAs encoded by the corresponding genomes. Translation factors required for protein synthesis in the apicoplast and mitochondrion have been reported in *P. falciparum*. The translation factors, including initiation, elongation, and termination factors, are coded by the nuclear genome, whereas the only elongation factor encoded by apicoplast genome is EF-Tu (Chaubey 2005). mRNA cap-binding by eIF-4E and interactions of eIF-4G with eIF-4E and other factors have been reported (Ruthenburg 2007). Most of the factors required for efficient protein synthesis and export have been annotated in the *Plasmodium* genome; however, their precise role and interaction with other factors are not yet confirmed.

Using co-immunoprecipitation to enrich for Maurer's cleft proteins, a novel subtelomeric gene family encoding 11 transmembrane proteins was identified (Sam-Yellowe 2004) by multidimensional protein identification technology (MudPIT). Most of these proteins were expressed and localized to the Maurer's cleft during the trophozoite stage. Furthermore, in a study to distinguish parasite proteins from host proteins, 78 proteins were found to be associated with Maurer's cleft (Vincensini 2005). Using MudPIT again, 138 proteins were identified from merozoite rhoptries from three different *Plasmodium* rodent species (Kaneko 2001; Sam-Yellowe 2004). Proteome analysis has also been performed to look at proteins exported to the infected cell surface (Florens 2004), identifying two novel surface proteins. These studies have highlighted the unique parasite proteome environment, suggesting important novel targets for drug discovery.

Proteome changes in response to drugs

The translation system has been an important target for malaria drug development. Owing to their prokaryotic origin, mitochondria and apicoplasts are the favored drug targets, and many translation-inhibiting drugs, including tetracyclines, target these organelles (Dahl 2006). Similar to transcription, differential proteomic responses have been observed in *P. falciparum* drug treatments. The proteome analysis provided additional information about the mode of action of drugs, especially in the absence of transcriptional response, as observed in case of antifolate inhibitors (Ganesan 2008). Similar upregulation of antifolate drug targets was observed in *P. falciparum* cells treated with the dihydrofolate reductase (DHFR) inhibitor WR99210 (Nirmalan 2004).

Prieto's group investigated the mode of action of two of the most important antimalarial drugs, artemisinin and chloroquine, by conducting a quantitative proteomics survey of *P. falciparum* parasites treated with these two drugs (Prieto 2008). Chloroquine treatment led to upregulation and downregulation of 41 and 14 proteins, respectively. The upregulated proteins included nuclear factors and proteins involved in translation. Artemisinin resulted in less-defined proteome changes, with only 38 and 8 proteins up- or downregulated, respectively.

Importantly, there was an upregulation of multidrug-resistant transporter PfMdr1, previously linked with drug-resistant phenotypes of *P. falciparum*, in both artemisinin- and chloroquine-treated cells. In response to two core compounds of the Coartem formulation artemether and lumefantrine (Makanga 2005), a relatively small number of proteins, including molecular chaperones as well as proteins involved in glycolysis and redox metabolism, were upregulated.

Doxycycline, an important drug in malaria chemoprophylaxis, was used to study protein expression changes in the schizont stage of *P. falciparum* (Briolant 2010). Doxycycline treatment followed by 2D-DIGE or isobaric tag for relative and absolute quantification (iTRAQ) resulted in deregulation of 32 and 40 *P. falciparum* proteins, respectively. The specific response to doxycycline included proteins linked with mitochondrial function. Interestingly, the doxycycline- and the Coartem-induced proteomic variations were also reflected at the mRNA level (Makanga 2005; Briolant 2010).

Specific small molecular inhibitors and other physiological perturbations also resulted in proteomic responses. The choline inhibitor compound T4 that identified only nonspecific transcriptional changes elicited much more specific responses at the protein level (Le Roch 2008). There was an increase in proteins associated with digestive vacuolar function and decrease in choline/ethanolamine phosphotransferase (PfCEPT, MAL6P1.145), which is involved in the final step of phosphoethanolamine synthesis, the direct target of this inhibitor. The combined transcriptomic and proteomic analysis of PfAdoMetDC/ODC co-inhibition resulted in changes in polyamine and methionine metabolism at both mRNA and protein levels (van Brummelen 2009).

It is clear from all the above data that it is crucial to include proteomic approaches in the studies of transcriptional regulation and growth perturbations because the final effect may be reflected in proteome change or transcriptional change, or both.

Conclusion

The functional genomic approaches have been of immense importance in elucidating some of the important aspects of the complex biology of *Plasmodium* parasites. The wide range of information collected from such high-throughput studies have revealed that a unique gene-regulation system in *Plasmodium* is observed during transcriptional, post-transcriptional, and translational control. These differences in molecular mechanisms from other eukaryotes have not only contributed to understanding of the parasite's biology but also are being exploited to design intervention strategies against this important human pathogen.

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CHAPTER 9

The biochemistry of *Plasmodium falciparum*: An updated overview

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The last overview of the biochemistry of the malaria parasite was provided by Irwin Sherman's monograph, which, adhering to his good tradition, summarized this important field for the community (Sherman 1979, 2009). However, this chapter essentially deals with metabolism as a set of chemical reactions organized in cellular metabolic pathways. The description of the various pathways relies heavily on the schemes of the website on the functional genomics of *Plasmodium falciparum*, Malaria Parasite Metabolic Pathways (MPMP, http://mpmp.huji.ac.il/). Each pathway will be described briefly, emphasizing the novelties of recent years.

MPMP

The MPMP project organizes data from the genome project of the parasite into assemblies with shared physiological and biological functions. It deals exclusively with the erythrocytic stage of *P. falciparum*. Metabolic pathways are shown in schemes of metabolite flows, which contain enzyme names, EC numbers (Enzyme Commission numbers), distinct biochemical reactions including all chemicals involved, and connections with other pathways. The schemes are extensively linked to other databases such as parasite genomics, biochemistry, and literature. It also contains information about gene expression and the subcellular location of gene products. Recently, all target-oriented drugs/inhibitors have been added to MPMP. Following the subdivisions of MPMP, this chapter deals with the metabolic pathways of carbohydrates, lipids, nucleotides, amino acids, redox metabolism, pathways in the mitochondrion, apicoplast, and hemoglobin (Hb) digestion.

Carbohydrates

Glycolysis

P. falciparum depends on glycolysis coupled with homolactic fermentation during the erythrocytic stages for energy production. No Pasteur effect could be identified. Glycolysis is a pathway consisting of 10 enzymes that converts glucose to pyruvate, but because the parasite's citric acid cycle uses only minimal amounts of pyruvate, the final product is lactic acid. The free energy released in this process is used to form the high-energy compounds ATP (2 moles/mole of glucose) and NADH. The pathway is depicted in Figure 9.1 in a broad context, showing the transporters responsible for glucose uptake

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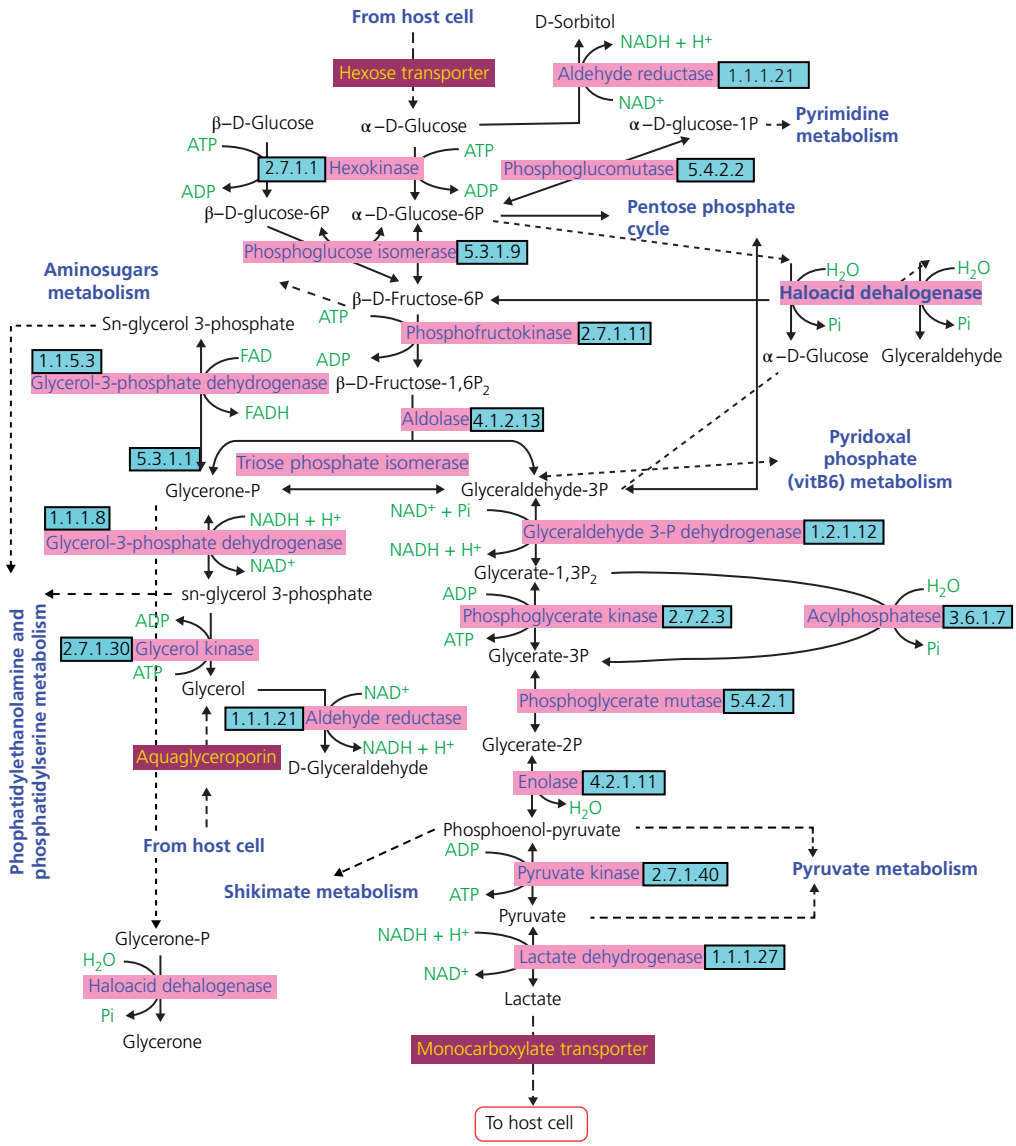


Figure 9.1 Glycolysis. The classical glycolytic pathway is shown with its different connections to other pathways. Also shown are the transport systems through which glucose is imported and lactate is exported and the pathways of glycerol uptake and production.

and release of lactate. Most of the lactate is expelled to the extracellular medium (Elliott 2001) and perhaps is the cause of lactic acidosis in severe malaria (Agbenyega 2000).

The parasite's glycolysis is up to 100 times more active than that of its host erythrocyte (Mehta 2005, 2006). Also shown are the connections with glycerol metabolism and other pathways. All genes encoding for glycolytic enzymes are found in the parasite genome with few paralogues. The biochemical properties of phosphofructokinase (PFK; EC 2.7.1.11) have been studied in *Plasmodium berghei*. Unlike the host enzyme, which is activated by AMP and inhibited by ATP/citrate, the parasite PFK could be the rate-limiting enzyme of the pathway (Mony 2009). Purified recombinant pyruvate kinase (PK; EC 2.7.1.40) exists as a homotetramer, and is activated by phosphoenolpyruvate and ADP but not by the general activator fructose-1,6-bisphosphate (Chan & Sim 2005). It is inactivated by pyridoxal-5′-phosphate, ATP, and citrate. Hexokinase (EC 2.7.1.1), aldolase (EC 4.1.2.13), and enolase (EC 4.2.1.11) are located in the cytosol, although hexokinase seems to preferentially bind with membranes (Olafsson 1992). The crystal structures of triose phosphate isomerase (Parthasarathy 2002a, 2002b), phosphoglucose isomerase (Aoki 2010), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), and aldolase (Kim 1998) have been reported (Robien 2006). In most cases, the crystal structures of the host enzymes were significantly distinct from the parasite enzymes (Cameron 2004), raising hopes for antimalarial drug design. Inhibitors have been identified that impede accumulation and phosphorylation of glucose by hexokinase, as well as pH regulation within the parasite cytosol. LDH recycles NADH to provide the constant supply of NAD^+ during glycolysis, and its inhibition is expected to inhibit glycolysis and ATP generation. Although no drug leads have so far emerged, the search for molecules inhibiting this pathway continues by using alternative techniques (Penna-Coutinho 2011).

Metabolic intermediates of glycolysis can be used by other pathways such as the pentose phosphate pathway (PPP), pyrimidine, shikimate, pyridoxal phosphate, phospholipids, and pyruvate metabolism, as indicated in Figure 9.1. However, their relative dependence for glycolytic intermediates is not known. During glycolysis, 2 moles of lactate are expected to be produced for each glucose molecule consumed, but a measurement of these parameters indicates that the ratio is actually 1:1.3 (Mehta 2005; Jensen 1983), implying that only about 50% to 60 % glucose uptake is used for ATP generation. All other uses of glucose and glycolytic intermediates do not seem to account for this discrepancy. A solution to this enigma was provided by the finding that the parasite produces glycerol (Lian 2009). Following 2 hours of incubation with [1-^{13}C] glucose, equimloar amounts of C-1,3 glycerol and C-3 lactate were detected. Authors suggested that this observation could have resulted from the metabolic adaptation to growth in a limited O_2 and elevated CO_2 environment through the glycerol-3-phosphate shuttle, as also is observed in yeast (see Mitochondrial electron flow, later). However, it could also happen through glyceraldehyde 3-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase (for the reoxidation of NADH) and further reverse action of glycerol kinase on glycerol-3-phosphate to produce glycerol. This glycerol should exit the infected cell, and only direct measurement of its release into the culture medium would confirm this hypothesis .

Interestingly, elevated levels of pyruvate and alanine were also found in the serum of *P. falciparum* malaria patients (Pukrittayakamee 2002). Although there may be a direct metabolic reason for the high alanine levels, the authors suggested that systemic alanine clearance is impaired in acute disease. It was also shown that parasites can supply ATP to the host cell whose glycolytic activity may be compromised (Bashan N 1975) due to a decrease in the host cell potassium concentration.

Pentose phosphate pathway

The major function of the pentose phosphate pathway (PPP) is the reduction of NADP to NADPH, which is used by the antioxidant defense mechanisms, the conversion of ribonucleotides to deoxyribonucleotides, and the provision of 5-phospho-D-ribosyl α-1-pyrophosphate (PRPP) for nucleoside biosynthesis (Figure 9.2).The PPP activity in the infected erythrocyte was found to be 78 times

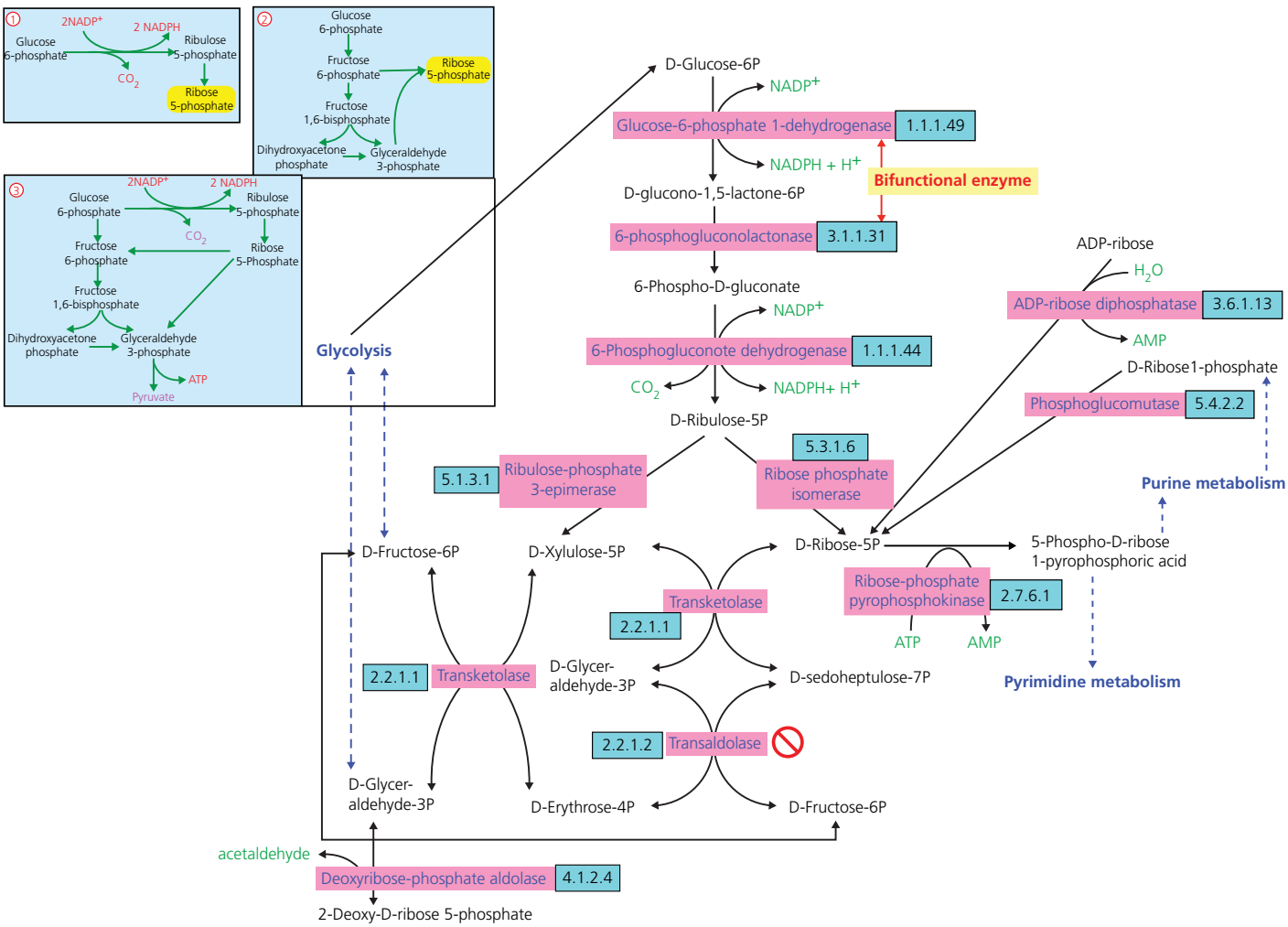


Figure 9.2 Pentose phosphate pathway (PPP). The pathway is a major producer of NADPH and of 5-phospho-D-ribose 1-pyrophosphoric acid (PRPP). The PPP can function in different modes depending on the needs of the cell. As shown in the inset, in mode 1, both ribose-5-phosphate and NADPH are needed. All the ribulose 5-phosphate is isomerized to ribose 5-phosphate, which is used for the synthesis of PRPP. In mode 2, more ribose-5-phosphate is needed than NADPH. Ribose 5-phosphate is synthesized by the non-oxidative arm using fructose-6-phosphate and glyceraldehydes-3-phosphate supplied by glycolysis. In mode 3, the cell needs NADPH and ATP but not ribose-5-phosphate. Ribulose-5-phosphate is converted to fructose-6-phosphate and glyceraldehydes-3-phosphate, which are channeled into glycolysis. Notice that no gene for encoding transaldolase could be found in the genome, indicated by the "no entry" icon.

higher than that in a normal erythrocyte (Atamna 1994). The parasite PPP activity constitutes 82% of the total activity observed in the intact infected erythrocyte (Atamna 1994). The first two enzymes of PPP are combined into a unique bifunctional enzyme named glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (EC 1.1.1.49; EC 3.1.1.31). The enzyme has been expressed in a heterologous system, and its kinetic parameters demonstrated remarkable functional and structural differences from the human enzymes, suggesting its potential use as a drug target (Clarke 2001; Jortzik 2011).

The function of PPP depends on the synthesis of $NADP^+$ and thiamine pyrophosphate, a coenzyme of the PPP enzymes transketolase (EC 2.2.1.1) and transaldolase (for which no encoding genes were found; EC 2.2.1.2). Based on stage-specific transcription (Bozdech and Ginsburg 2005), it was found that the expression of the PPP enzyme genes is not coordinated and the expression of the last enzyme determines the timing of the full deployment of the pathway's activity. It seems that the activity of PPP involves only the oxidative arm of PPP that is geared for maximal $NADP^+$ reduction and ribose-5-phosphate production (see Figure 9.2) during the early stages of parasite development.

The synthesis of thiamine diphosphate occurs much later than the expression of transketolase. Later in the parasite cycle, the non-oxidative arm of PPP that can use fructose-6-phosphate and glyceraldehyde-3-phosphate supplied by glycolysis becomes fully deployed, allowing the maximum production of ribose-5-phosphate. These apparent discordances were addressed experimentally: D-[1-^{14}C]glucose and D-[6-^{14}C]glucose were found to equally contribute to newly synthesized nucleic acids, meaning that ribose-5-phosphate needed for this synthesis is contributed by the non-oxidative activity of PPP (Atamna 1994). Thus, a major portion of parasite PPP activity and the activated PPP of the host cell are devoted to counteracting the endogenously generated oxidative stress. But because the glycolytic flux is much higher than PPP activity, there is plenty of fructose-6-phosphate and glyceraldehyde-3-phosphate produced by the former to get the non-oxidative arm to produce PPRP.

Pyruvate metabolism

Pyruvate metabolism is divided among the cytosol and the relict plastid, the apicoplast (Figure 9.3). The cytosolic aspect of pyruvate metabolism involves the actions of PEP carboxykinase (EC 4.1.1.49), which accounts for the CO_2 fixation (Blum and Ginsburg 1984) to produce oxaloacetate. It is closely related to PEPCK from plants (Hayward 2000). The *P. falciparum* genome also contains a gene that encodes a PEP carboxylase (PEPC, EC 4.1.1.31). In *P. berghei* the enzymatic reaction catalyzes the conversion of PEP and carbon dioxide to oxaloacetate (McDaniel & Siu 1972). Oxaloacetate can be used by malate dehydrogenase (MDH; EC 1.1.1.37; Lang-Unnasch 1992). The *P. falciparum* MDH has been cloned, its crystal structure has been analyzed, and the biochemical properties were partially characterized (Tripathi 2004).

The activity of MDH can assist that of LDH for the regeneration of NAD^+ and thus produce malate. However, MDH could work in reverse: Malate generated by the mitochondrial citric acid (TCA) cycle (see below) is oxidized to produce oxaloacetate. The latter will be used by aspartate transaminase (EC 2.6.1.1) to provide 2-oxoglutarate for the mitochondrial TCA cycle, entering the mitochondrion through the malate:2-oxoglutarate antiporter. This enzyme could also work in the reverse direction using 2-oxoglutarate to produce oxaloacetate. Metabolomic investigations have shown that parasites fed with uniformly ^{13}C-labeled glucose generated oxaloacetate/malate labeled at three of the four carbons, indicating PEP carboxylation (Cobbold 2013; McRae 2013). All these options pose some intriguing stoichiometric problems that await resolution. The exchange of malate for 2-oxoglutarate could essentially provide $NADH^+$ to the mitochondrial matrix (see the inset in Figure 9.34) to be used by malate:quinone oxidoreductase to reduce ubiquinone. Glycolysis can also provide pyruvate directly to the TCA cycle (Cobbold 2013; MacRae 2013).

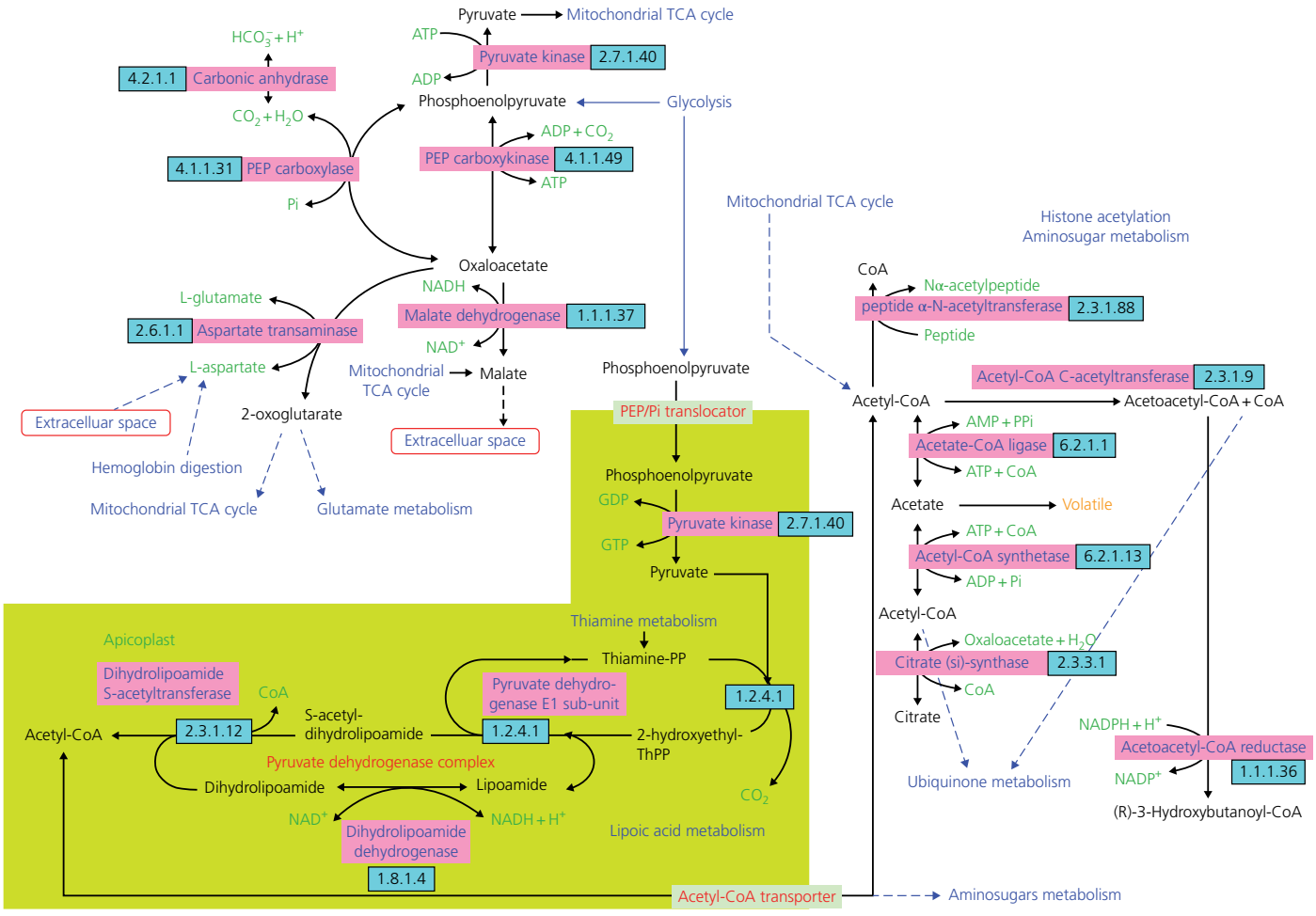


Figure 9.3 Pyruvate metabolism. The compartmentation of pyruvate is emphasized where all reactions appearing in the apicoplast are shown on a green background. While the apicoplast produces acetyl-CoA from phosphoenolpyruvate, the mitochondrion uses α-ketoglutarate for this purpose (see Figure 9.34). Acetyl-CoA is used both for protein acetylation (mostly histones) and for the synthesis of amino sugars (see Figure 9.5).

Pyruvate metabolism is the main source for energy in the apicoplast. As seen in Figure 9.3, phosphoenolpyruvate produced by glycolysis is translocated into the apicoplast to provide for the synthesis of ATP and GTP, yielding pyruvate, which is converted by pyruvate dehydrogenase (PDH) to acetyl-CoA, the major precursor for type II fatty acid synthesis (FAS II). PDH complexes are composed of four subunits: E1α, E1β, E2, E3. Like most other apicoplast proteins, these subunits are encoded by the nuclear DNA and are targeted to the apicoplast by means of bipartite N-terminal leader sequences. The recombinantly expressed catalytic domain of the PDH subunit E2 showed high enzymatic activity *in vitro,* indicating that pyruvate is converted to acetyl-CoA in the apicoplast, for use in fatty acid biosynthesis (Foth 2005). Acetyl-CoA produced in the apicoplast is exported out of the organelle and serves acetylation of histones and amino sugars (Cobbold 2013). Phosphoenolpyruvate and glycerone-phosphate produced by glycolysis enter the apicoplast and serve as substrates for the biosynthesis of isoprenoids.

Glyoxalase metabolism

The features of the glyoxalase metabolic pathway have been reviewed (Urscher 2011). Intensive glucose metabolism results in spontaneous formation of toxic methylglyoxal (Figure 9.4) from the glycolytic intermediate glycerone phosphate (dihydroxyacetone-P). 2-Oxoaldehydes can also be produced from acetone and glycerol degradation or lipid peroxidation, but the genes encoding the necessary enzymes are absent from the genome. Methylglyoxal is detoxified through the serial action of glyoxalase I (Glo1, (EC 4.4.1.5) and glyoxalase II (Glo2, EC 3.1.2.6) and the cofactor glutathione to yield D-lactate, whose production and excretion from infected cells is about 30-fold higher than that of uninfected erythrocytes (Vander Jagt, 1990). An orthologue of Glo2 is located in the apicoplast, but its function is unknown. The biochemical properties of Glo1 and Glo2 have been studied in some detail, specifically the allosteric regulation of Glo I (Iozef 2003; Deponte 2007). Various efforts to develop inhibitors for both these enzymes have so far not been successful (Akoachere 2005; Urscher and Deponte 2009).

Aminosugar metabolism

An amino sugar is a sugar in which one or more non-glycosidic hydroxyl groups are replaced by an amino or substituted amino group. An abundant example is D-glucosamine. Amino sugars are important constituents of glycoproteins and oligosaccharides involved in biological recognition. Essentially nothing is known about the details of amino sugar biosynthesis in malaria parasites or about the subcellular compartment in which it occurs. Probably the most significant metabolic product of this pathway is UDP-*N*-acetyl-D-glucosamine, which is the major substrate for glycosylphosphatidyl-inositol (GPI) biosynthesis, which is described later in this chapter. Protein glycosylation (Figure 9.5) was demonstrated using radiolabeled glucosamine, fucose and mannose (Udeinya and Van Dyke 1980). That this not only reflects GPI anchor synthesis is evident by the ability of the glycoproteins to bind Con A, a wheat germ agglutinin (Ramasamy 1987).

The existence of *N*-glycosylation in *Plasmodium* has been controversial. Some investigators identified a 14-sugar *Plasmodium N*-glycan resembling that of the human host (Kimura 1996), but others failed to identify any *N*-glycans (Berhe 2000; Gowda 1997). Both *Plasmodium* and *Toxoplasma* are missing *N*-glycan–dependent quality-control factors involved in the folding of glycoproteins (Banerjee 2007). It has been shown that early blood stage parasites make lipid-linked *N*-glycan precursors that are composed predominantly of a single *N*-acetyl-D-glucosamine (GlcNAc), while later blood-stage parasites make *N*-glycan precursors that are a mixture of GlcNAc and GlcNAc2. Finally, it should be underscored that the parasite genome lacks almost all the genes encoding

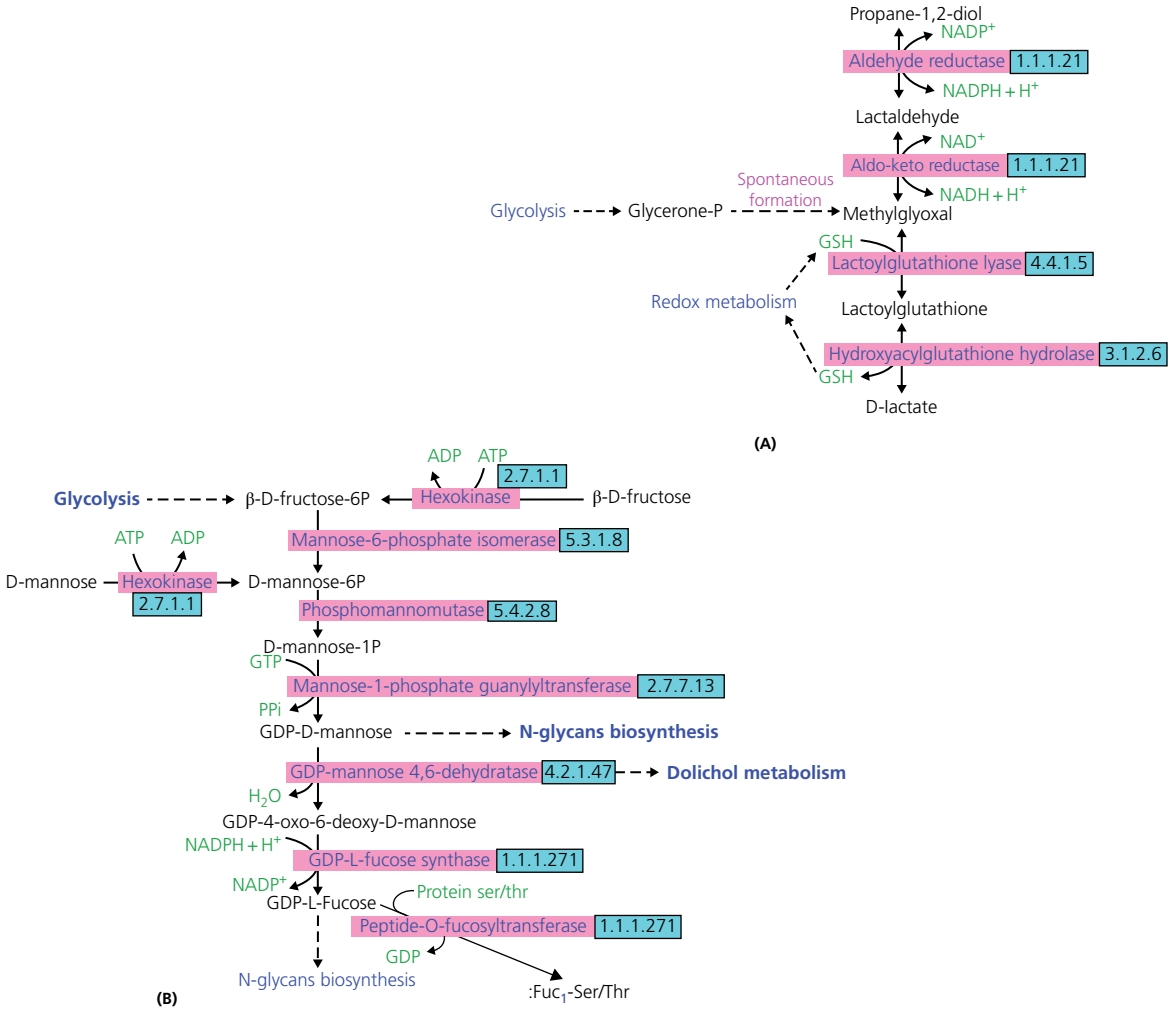


Figure 9.4 Glyoxalase and mannose metabolism. *A,* the glyoxalase pathway detoxifies methylglyoxal, which is formed during high rates of glycolysis. The upward path from methylglyoxal is supported by the presence of encoding genes but not by experimental evidence. *B,* Mannose is supplied either from glucose or from the extracellular space. Its products are used eventually for the synthesis of GPI anchors (Figure 9.12) after combining with dolichol (Figure 9.11). Although products can also be used for glycan synthesis, this process is debatable (see text).

glycosyltransferases involved in dolichol-PP attachment, which is an essential requirement for protein N-glycosylation.

O-Glycosylation in *P. falciparum* has been investigated by several workers (Dayal-Drager 1991; Nasir-ud-Din 1992; Macedo 2010). Mucin-type *O*-glycans were not detected in the latter studies, consistent with the failure to detect synthesis of GalNAc or epimerization of GlcNac to GalNac. Radiolabeling with GalN demonstrated that this precursor was mostly phosphorylated, but GalNAc, GlcNAc, and the corresponding sugar nucleotides were not detectable. These results are consistent with previous data suggesting that *Plasmodium* lacks the ability to epimerize GalN into GlcN and *vice*

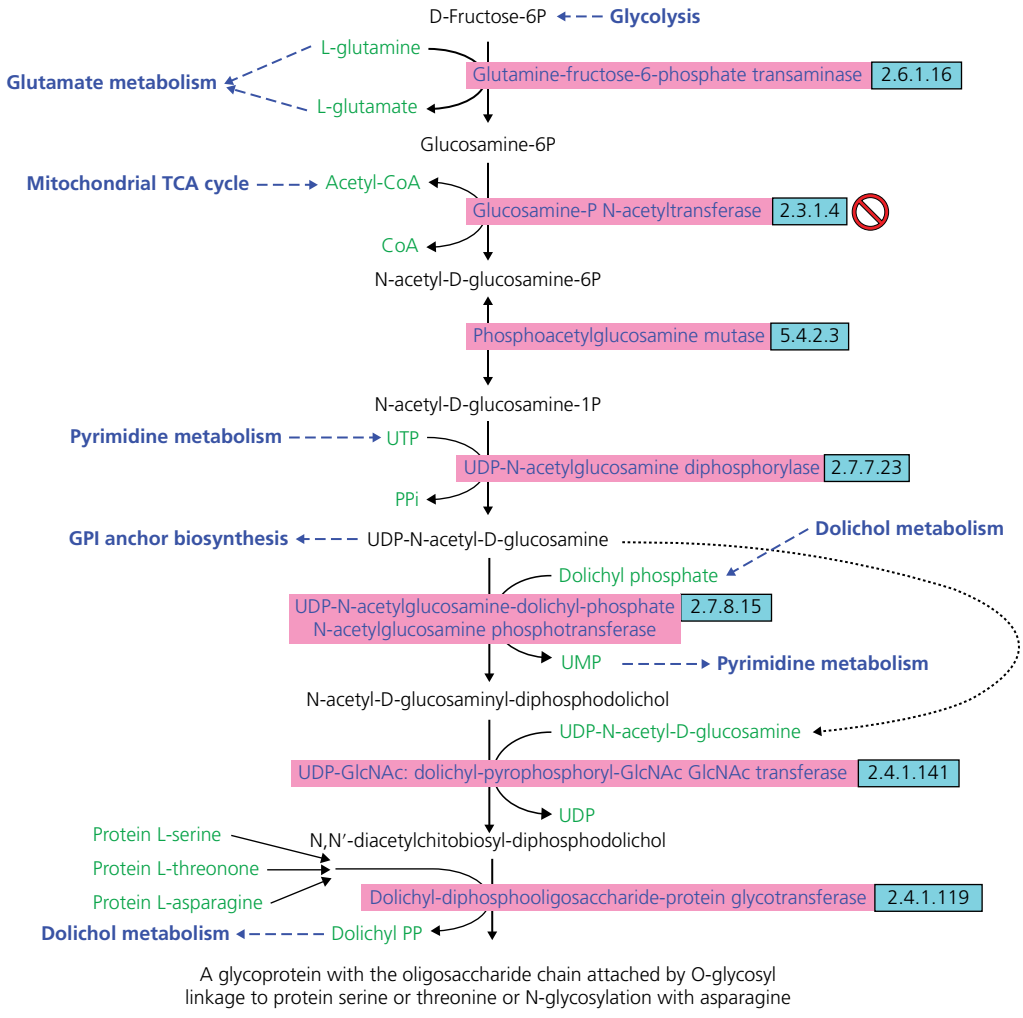


Figure 9.5 Aminosugar metabolism. Amino sugars are synthesized as precursors for the generation of GPI anchors and for *N*- and *O*-glycosylations of proteins. The "no entry" sign indicates that a gene encoding this enzyme could not be found in the genome by sequence homology.

versa (Macedo 2010); The presence of *O*-linked glycoproteins during the schizont stage has been confirmed (Kimura 1996). However, very little is known about the roles of glycosylation on the function(s) of the parasite proteins.

Lipid metabolism

Fatty acid biosynthesis

Fatty acid synthesis (FAS) is a fundamental aspect of cell physiology. There are two basic types of FAS architecture. The prototypical FAS I is found in mammals and FAS II is found in bacteria, plants, and parasites. The FAS II pathway of *P. falciparum*, as shown in Figure 9.6, takes place

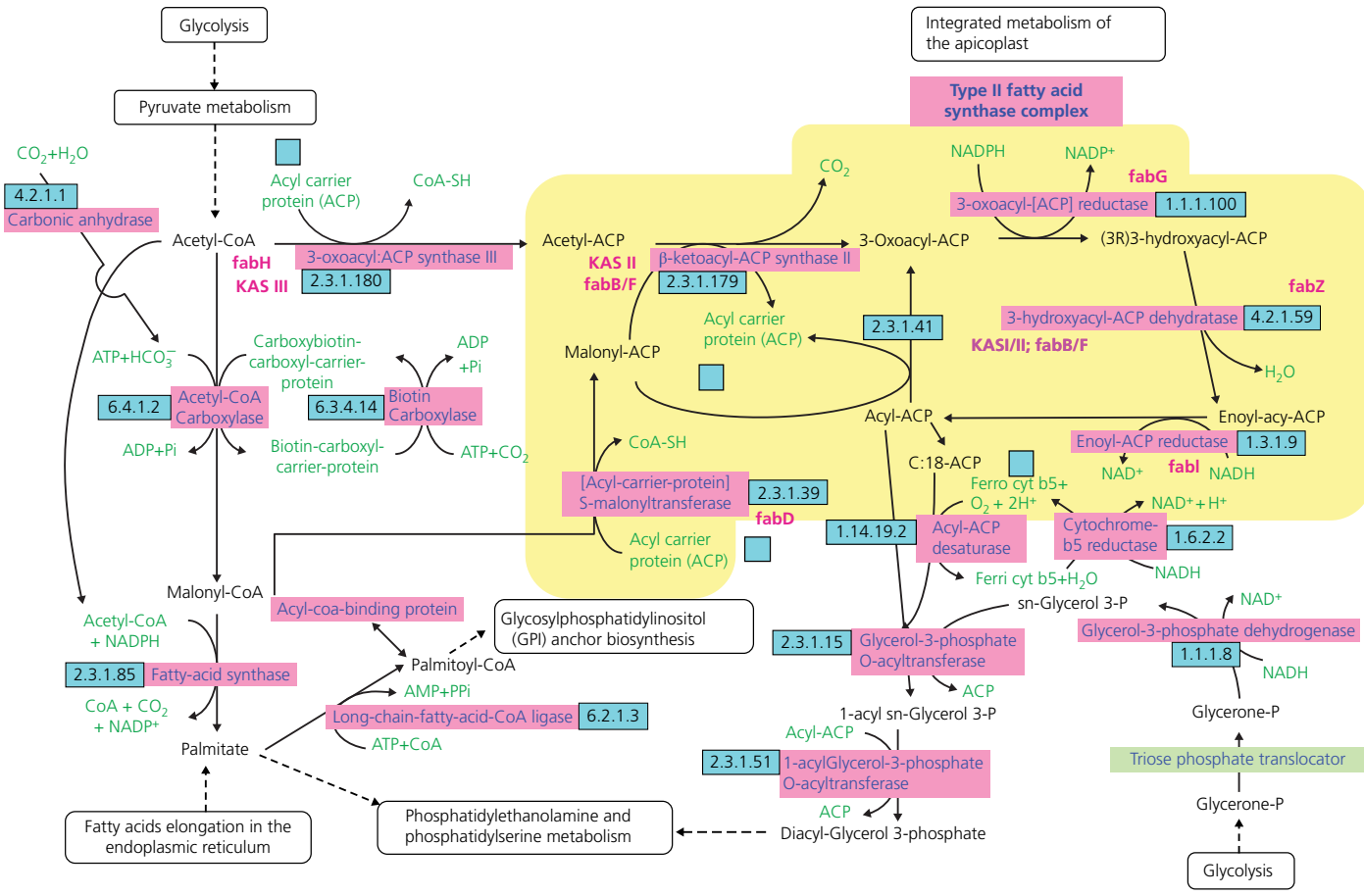


Figure 9.6 Fatty acid synthesis in the apicoplast. All this intricate pathway is located in the apicoplast, receiving inputs from different other pathways. The fatty acid type II synthase complex is shown on a yellow background. Also shown are some of the known inhibitors in red (cerulenin, fenoxaprop clonidafop, thiolactomycin, triclosan) of particular enzymes.

essentially in the apicoplast (Lim and McFadden 2010). It is supplied by PEP, which is converted to acetyl-CoA (see the earlier discussion of pyruvate metabolism) and then to malonyl-CoA by acetyl-CoA carboxylase (EC 6.4.1.2). The elongation of the fatty acid is accomplished by rounds of priming acyl carrier protein with a malonyl moiety, which adds two carbons to the elongating acyl chain in each cycle of successive condensation, reduction, dehydration, and reduction reactions.

Activity of β-ketoacyl-ACP synthase (EC 2.3.1.41) was maximal with C4:0- to C10:0-acyl-ACPs and declined when longer-chain acyl-ACPs were synthesized (Lack 2006). Longer fatty acids are scavenged from the extracellular space/serum. Additional enzymes undertake desaturation and glyceride synthesis. Products essentially serve as substrate for GPI anchor, and phospholipid and isoprene syntheses, but the means for their excretion are yet not yet known. All enzymes are encoded by the nuclear genome. Partial biochemical characterization of enoyl-ACP reductase (ENR, EC 1.3.1.9) has been reported (Kapoor 2001). As evidenced by several studies, deletion of ENR indicates that the pathway is only essential in liver stages of *Plasmodium yoelii* (Vaughan 2009) and *P. berghei* (Yu 2008). This stage-dependence of FAS II activity can be rationalized on high demand for membrane synthesis precursors during highly prolific liver stage. Considering that in liver-stage schizogony, each sporozoite gives rise to tens of thousands of merozoites, the necessity of the apicoplast FAS II pathway for liver-stage replication becomes evident (Prudencio 2006).

The crystal structure and biochemical properties of 3-oxoacyl-ACP reductase (EC 1.1.1.100) have been investigated (Wickramasinghe 2006). Enoyl-ACP reductase (EC 1.3.1.9) was also studied quite intensively for its specificity toward the specific inhibitor triclosan (Suguna 2001; Perozzo 2002; Kapoor 2001). However, it was later conclusively demonstrated that triclosan does not act on ENR (Yu 2008) but has a rather nonparasite-specific off-target effect (Lizundia 2009).

Long-chain fatty acid CoA synthetase (EC 6.2.1.3) is a large family (14 orthologues) of gene products. One of the orthologues (PF14_0761) is exported to the host cell cytosol and might participate in the construction of a tubulovesicular membrane system that connects the parasitophorous vacuole membrane (PVM) to the erythrocyte membrane (Matesanz 1999). The other orthologues are differentially expressed at different developmental stages, but their precise role is not known (Matesanz 2003). Importantly, an investigation has concluded that type II fatty acid synthesis is essential only for late liver stages of the rodent parasites and that deletion of enoyl-ACP reductase in the human malaria parasite *P. falciparum* does not show a reduction in *in vitro* asexual blood stage replication, implying that intrinsic fatty acid synthesis is needed only for a specific life cycle transition from liver to blood stages (Vaughan 2009).

Other sources of fatty acids are diacylglycerols and triacylglycerols, which are taken up from the extracellular space after erythrocyte invasion (Nawabi 2003). Triacylglycerol is a major lipid species taken up and stored in lipid droplets in late erythrocytic stages to be eventually used for phospholipid and GPI synthesis. The droplets were a few hundred nanometers in size and were seen closely associated with the digestive vacuole (Jackson 2004). Relatively large amounts of esterified monohydroxy derivatives and products of peroxidation of polyenoic fatty acids were found in infected cells, which increased with parasite maturation. The peroxidative products consisted of a complex mixture of monohydroxy derivatives of arachidonic (20:4(ω-6)) and linoleic acid (18:2(n-6)). Peroxidation was not enzymatic because no genes encoding lipoxygenase or lipoxygenase activity could be detected. Heme-catalyzed peroxidation was suggested to account for a large number of positional and optical isomers in racemic mixtures that were generated by incubation of arachidonic acid with hemozoin (Schwarzer 2003). The entire pathway of mono-, di-, and triglyceride synthesis can be seen in the MPMP map "Utilization of phospholipids" (http://mpmp.huji.ac.il/maps/utilPhospho.html).

Phosphatidylcholine

Parasite growth and multiplication during the erythrocytic stages involves membrane synthesis and recycling. Together with the altered phospholipid composition of the infected cell (Hsiao 1991; Prudencio 2006; Vial and Ben Mamoun 2009) it implies intensive phospholipid synthesis. Phosphatidylcholine (PC) is the major phospholipid in *P. falciparum* membranes, representing 57% in the parasite membrane and 39% in total infected erythrocyte as compared to 32% in uninfected erythrocytes. PC can be taken up by infected cells from extracellular medium (Simoes 1991).

PC is synthesized *de novo* by CDP-choline (Kennedy) pathway (Figure 9.7). It obtains choline from extracellular space through a vestigial erythrocyte choline transporter and new permeability pathways and then through a poly-specific organic cation transporter of the parasite membrane (Biagini 2004; Lehane 2004). Subsequently, choline is phosphorylated by choline kinase (EC 2.7.1.32) and then attached to CTP to generate CDP-choline by the pathway's rate-limiting enzyme choline-phosphate cytidylyltransferase (EC 2.7.7.15) and finally converted to PC by diacylglycerol choline phosphotransferase (EC 2.7.8.2) using diacylglycerol. The second pathway is the serine-decarboxylase phosphoethanolamine methyltransferase (SDPM) pathway, so called because serine obtained from Hb digestion is decarboxylated to ethanolamine and metabolized to phosphatidylethanolamine (PE). PE is transformed to phosphocholine by three consecutive methylation steps mediated by *S*-adenosylmethionine–dependent methyltransferase (PfPMT, EC 2.1.1.103) (Pessi 2004, 2005; Bobenchik 2011). PfPMT is located in the Golgi apparatus and when disrupted causes delayed growth and multiplication and increased cell death (Witola 2008), and it is essential for parasite gametocytogenesis and malaria transmission (Bobenchik 2013). Inspection of relative contribution of the two pathways indicated that 63% of PC was produced from the CDP-choline route, 9% of PC originated from the PE methylation path, and 28% was probably obtained from the host cell (Enjalbal 2004).

The use of enzymes of PC biosynthesis as drug targets has been reviewed (Ben Mamoun 2010). In fact, since the sequencing of the *P. falciparum* genome, PC biosynthesis is so far the only parasite target that has been successfully inhibited by drugs that have reached advanced stages of clinical trials (Nicolas 2005). PMT encoding genes and activity are missing in murine parasite species (Dechamps 2010). The various routes of PC degradation and interactions with other metabolic pathways are also shown in Figure 9.7.

Phosphatidylserine and phosphatidylethanolamine

Phosphatidylethanolamine (PE) is the second most abundant phospholipid in parasite membranes. It constitutes 27% of the parasite membranes, and phosphatidylserine (PS) contributes 6% (Hsiao 1991). As seen in Figure 9.8, PE can be generated either by the phosphatidylserine decarboxylase (EC 4.1.1.65) pathway or the CDP-ethanolamine alternative pathway. The relative contribution of the two pathways or the physiological conditions that favor action of one or the other are not known. Ethanolamine kinase (EC 2.7.1.82) and choline kinase (EC 2.7.1.32) enzymes are cytoplasmic, and their expression increases with parasite maturation. Although there was some overlap in specificity for their substrates, they could be inhibited by specific compounds. However, both enzymes were equally inhibited by bis-thiazolium compound T3 in correlation with impairment of cellular phosphatidylcholine biosynthesis.

Phosphatidylserine (PS) constitutes only 4% of the total parasite phospholipids and 12% in the uninfected erythrocyte, indicating *prima facie* some utilization/conversion of PS into other metabolites useful for the parasite. Infected erythrocytes were shown to incorporate radiolabeled serine into PS, PE, and PC. Serine was also incorporated into ethanolamine and phosphorylethanolamine by direct decarboxylation. These two metabolites accounted for 60% of the total radioactive

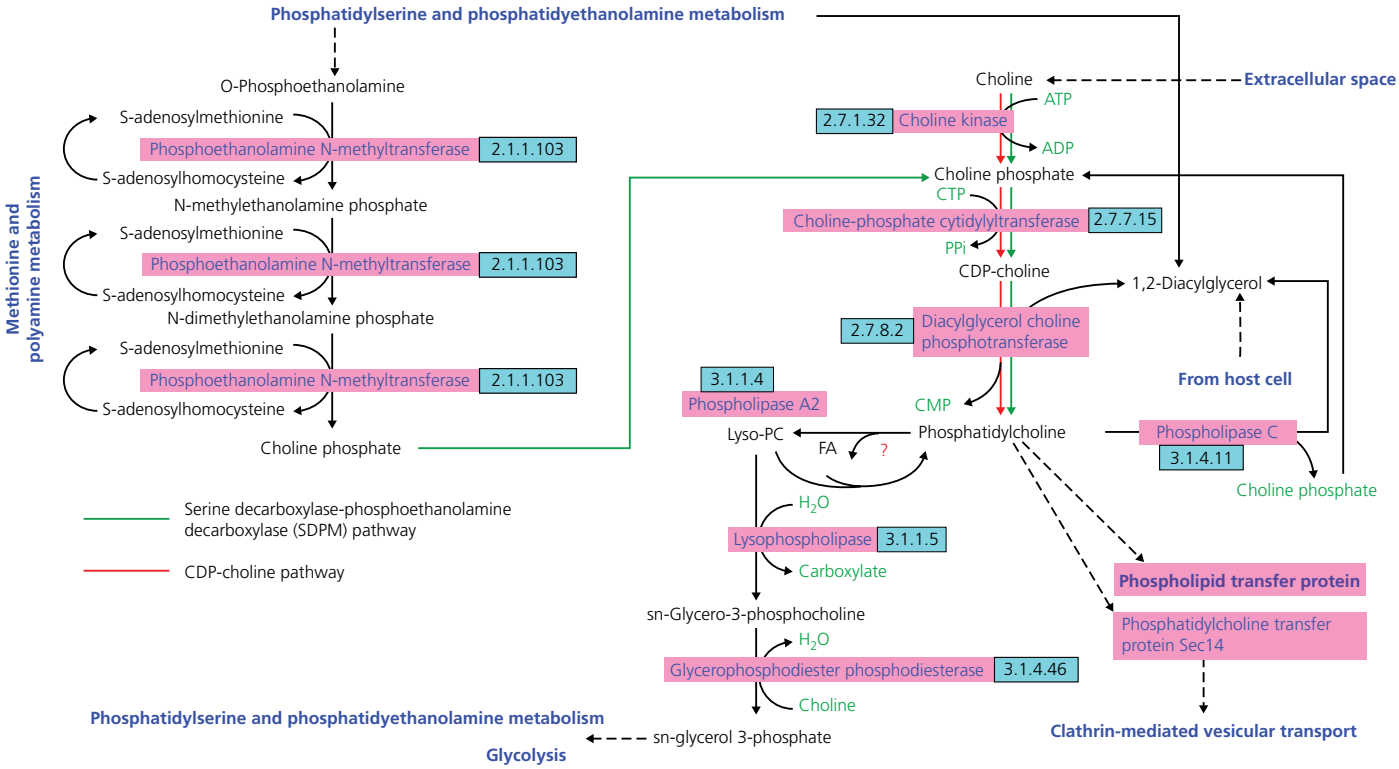


Figure 9.7 Phosphatidylcholine (PC) metabolism. PC is the major constituent of parasite membranes. The two alternative pathways for PC generation are shown – follow the green and red lines. Also shown are some of the PC degradation products and their recycling.

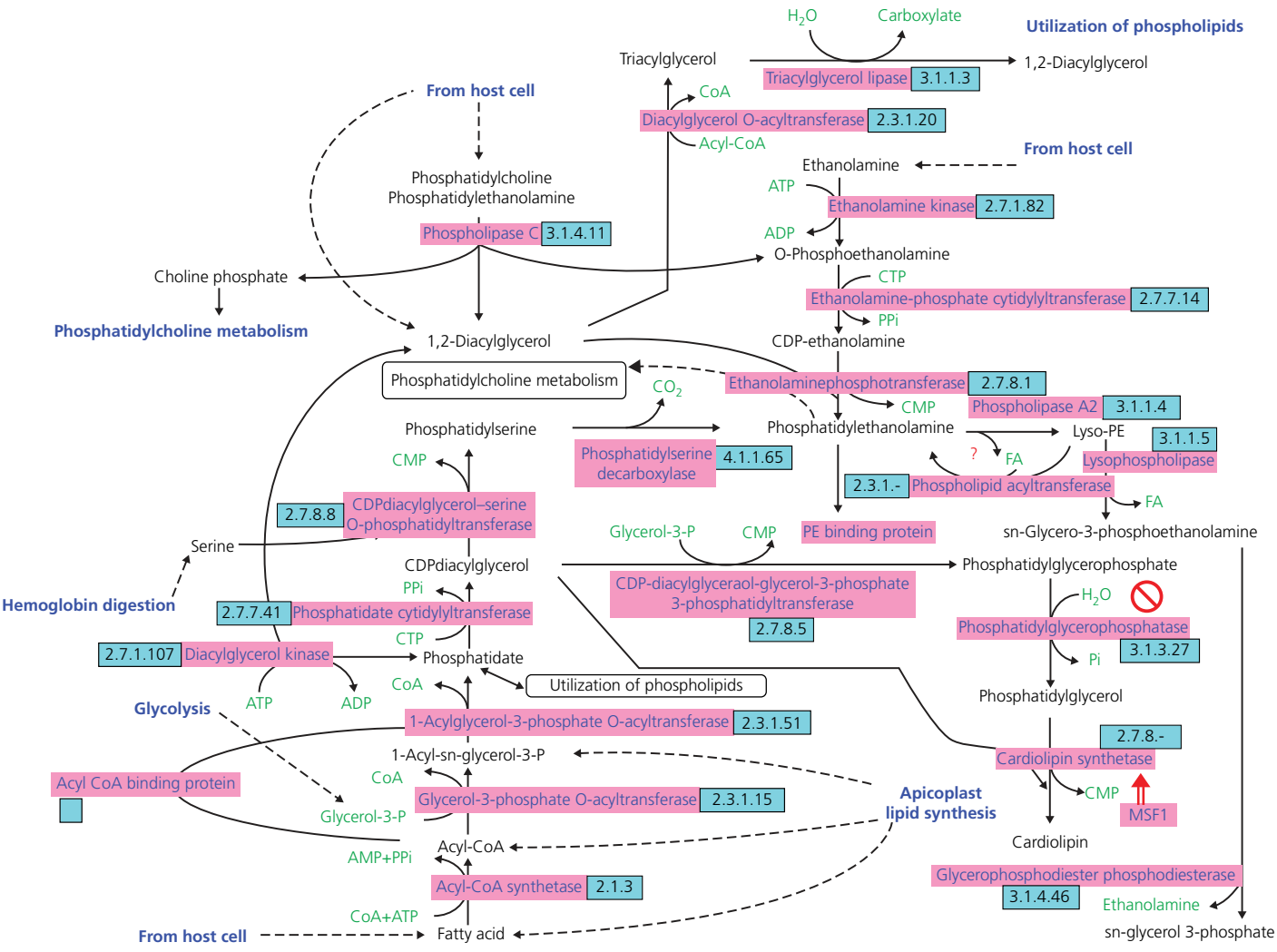


Figure 9.8 Phosphatidylethanolamine (PE) and phosphatidylserine (PS) metabolism. PE can be synthesized directly from ethanolamine or via the decarboxylation of PS. The intermediate CDP-diacylglycerol is also the source for the synthesis of cardiolipin, a typical mitochondrial membrane phospholipid.

water-soluble metabolites, although a serine decarboxylase encoding gene could not be found in the parasite genome. Thus, PS, whether obtained from the host cell or synthesized *de novo,* is a major precursor for generation of PE and subsequently PC.

Figure 9.8 also displays generation of phosphatidylglycerol (PG) and cardiolipin, a mitochondrion-specific phospholipid that constitutes 5.5% of the parasite's lipids. The path is nevertheless discontinuous because no gene could be identified to encode phosphatidylglycerolphosphatase (EC 3.1.3.27). However, it has been demonstrated that a mitochondrial protein tyrosine phosphatase in mammalian cells (PTPMT1) can mediate cardiolipin formation bypassing the phosphatidylglycerol intermediate (Zhang 2011). The genome of *P. falciparum* has three genes encoding proteins with this function (MAL13P1.168, PF13_0027, and PF11_0139), but none of them has a mitochondrial targeting signal. Finally, MSF1 has been shown to maintain proper mitochondrial electron transport chain function, respiratory competency, and the levels of cardiolipin (Hall 2011).

Neither PS nor PE is arranged symmetrically on the two leaflets of membranes. Phospholipid flippases in type IV P-type ATPase family (P4-ATPases) are essential components of plasma membrane, Golgi, and endosomal systems, playing critical roles in membrane biogenesis. These pumps flip phospholipid from the exoplasmic side across the bilayer, enriching the cytosolic leaflet with PS and PE. The P4-ATPases also help form transport vesicles that bud from Golgi and endosomal membranes, thereby affecting the composition of the membrane load (Lopez-Marques 2011; Witola 2008). The genome of *P. falciparum* has four genes encoding flippase (PF3D7_0319000, PF3D7_1219600, PF3D7_1223400, PF3D7_1468600). Also found in the genome is a gene encoding CDC50 (PF3D7_1133300), which may be essential for flippase action.

Phosphatidylinositol

Phosphatidylinositol constitutes 2.7% of the parasite membranes. Apart from being a membrane component, phosphorylation of the inositol head group by specific kinases results in the manufacture of different phosphoinositide species associated with discrete cellular compartments. They participate in various cellular processes, notably in signal transduction, cytoskeletal reorganization, cell motility, membrane traffic, DNA synthesis, cell cycle control, and adhesion (Di Paolo and De Camilli 2006). All enzymes shown in Figure 9.9 have genes encoding for them, but not all metabolic intermediates could be found. A detailed phosphoinositide profile of *P. falciparum*–infected erythrocytes revealed substantial quantities of 1-phosphatidyl-1D-*myo*-inositol 3-phosphate (PI3P), which were associated with the food vacuole membrane and apicoplast (Tawk 2010). PI4P, PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 were also detected. The pathway leading to these compounds and others is depicted in Figure 9.9.

Phosphatidylinositol 3-kinase activity was found to be essential for the parasite, being involved in endocytosis from the host and trafficking of Hb-laden vesicles to the food vacuole for export to the host cell cytosol (Vaid 2010). Concomitantly, PI3P was found to be a key ligand for conscription of endosomal regulatory proteins in higher eukaryotes. A selective PI3P binding zinc-finger motif, the FYVE domain family, is represented in the *P. falciparum* genome by a single gene and is involved in endocytosis of host cell cytosol (McIntosh 2007). The activity of the *P. falciparum* phosphatidylinositol 4-kinase was evidenced by complementation studies in *Saccharomyces cerevisiae* (Kruger 2010).

In addition to its role as a structural membrane component, sequential addition of sugar residues to PI by the action of glycosyltransferases in endoplasmic reticulum (ER) leads to synthesis of GPI, which serves as a membrane anchor for parasite surface proteins (Sanders 2006). In Figure 9.9, steps are also shown toward the synthesis of tetra- and pentakisphosphate. Although they have important roles in mammalian immune systems, absence of their substrate (D-*myo*-inositol 1,4,5-P_3)

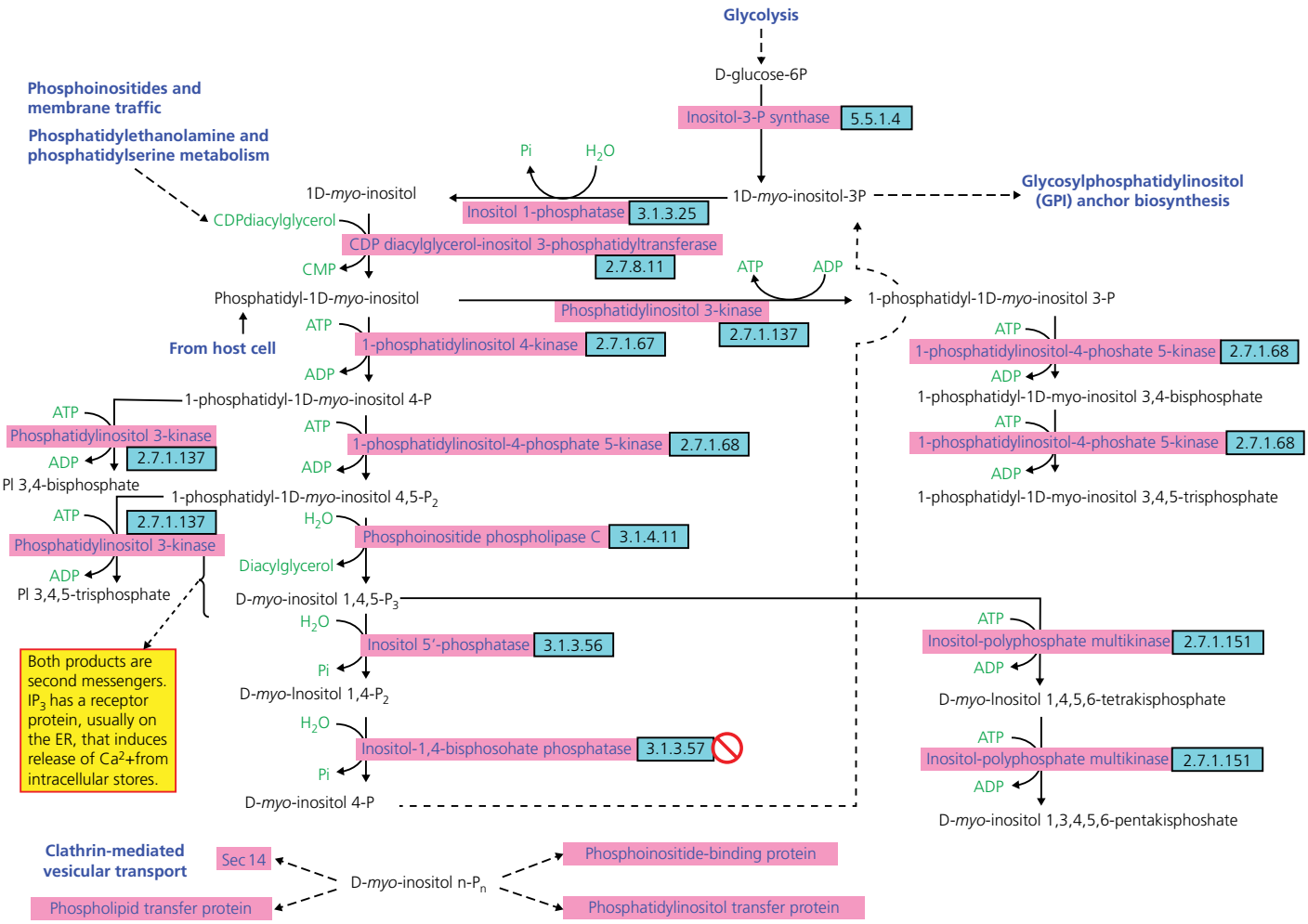


Figure 9.9 Inositol phosphate metabolism. Inositol can be phosphorylated to various levels, and each product can serve a specific role. Products serve in the synthesis of GPI anchors, in the endocytosis of host cell cytosol, and probably in other, as yet unraveled, signaling pathways.

in infected cells and lack of homologous roles in the parasite suggest that this pathway is not present in *P. falciparum*, but further investigations seem warranted.

Sphingomyelin

Sphingomyelin (SM) constitutes 5.7% of the parasite membrane and 14.6 % of the total lipids in the infected erythrocyte. Normal erythrocytes comprise 28% SM, thus indicating a considerable decrease of SM upon infection. An accumulation of extrinsic ceramide has been observed in the sites of SM synthesis in the infected erythrocyte, the intraerythrocytic cisternae, and the tubular membranes that appear to emerge from the parasitophorous vacuole (Haldar 1991). The synthesis of SM (Figure 9.10) has been located to the *cis*-Golgi, but unlike other Golgi proteins associated with

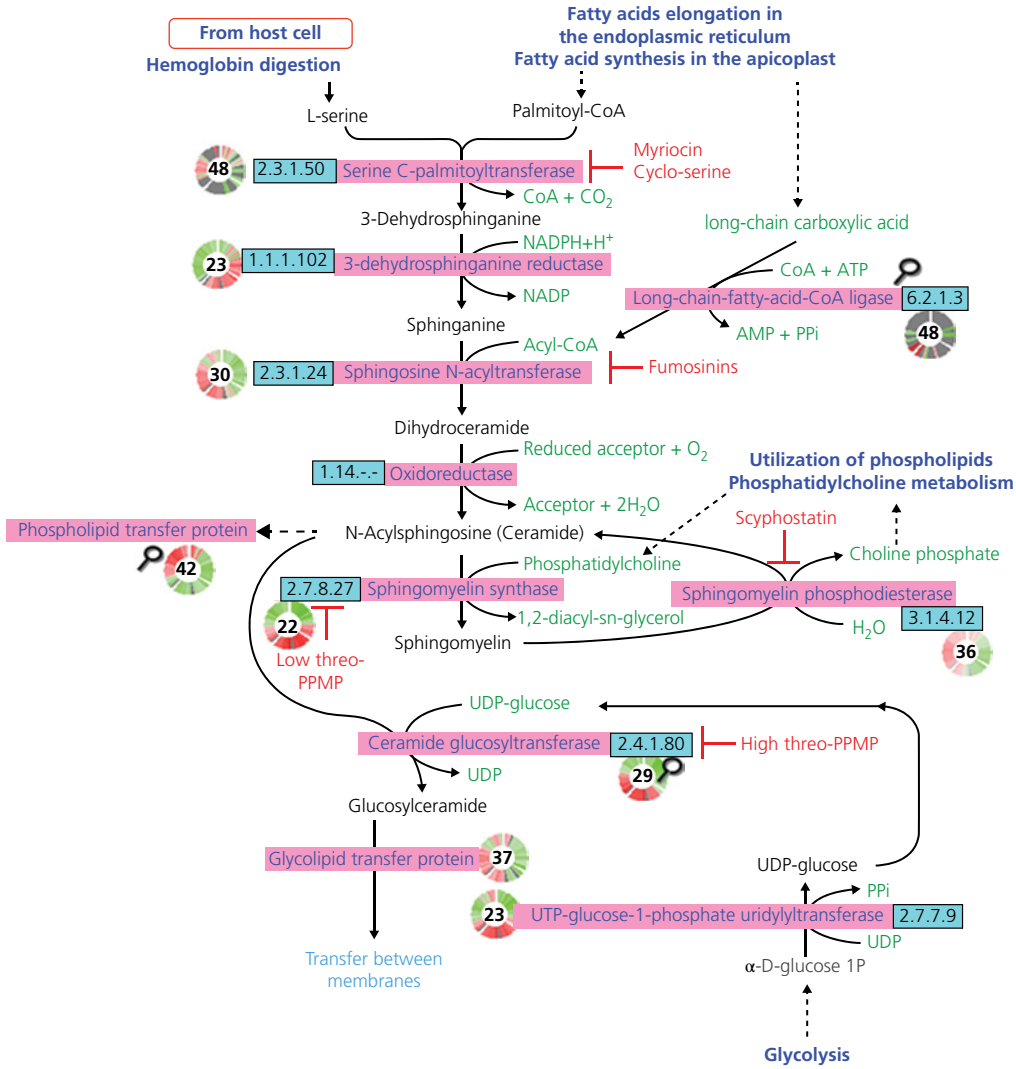


Figure 9.10 Sphigomyelin (SM) and ceramide metabolism. Sphingomyelin can be obtained from the host cell membrane, but the parasite has the full complement of enzymes that could synthesize SM *de novo* as well as for its conversion ceramide. SM is instrumental in the genesis of the Golgi apparatus and the tubulovesicular membrane (TVM).

this compartment, it does not reorganize upon Brefeldin-A treatment that is known to disrupt the Golgi (Elmendorf and Haldar 1993).

Evidence has been presented that in the extracellular merozoite stage, the parasite retains a SM synthase (EC 2.7.8.27) within its plasma membrane. However, during the following intracellular ring- and trophozoite-stages, a sizeable fraction (~26%) of SM synthase activity gets exported to tubulovesicular membranes (TVMs) in the host cell cytosol, where *de novo* synthesized SM is known to accumulate (Elmendorf and Haldar 1994). It has been suggested that SM is required for budding of the TVM from the PVM and for sorting of exported proteins from the TVM to the Maurer's clefts. Inhibition of SM synthase disrupts the TVM and inhibits parasite growth, indicating an essential role of TVM in parasite proliferation by facilitating nutrient and host protein uptake (Lauer 1995, 1997).

An ancillary role for SM was also revealed when it was found to be associated with detergent-resistant domains (along with cholesterol) in the PVM formed upon merozoite invasion. This SM is probably released from the invading merozoite and determines the assortment of host cell membrane proteins that enter the PVM (Lauer 2000). Sphingomyelin phosphodiesterase (EC 3.1.4.12) is a membrane-bound, Mg^{2+}-dependent, and neutral sphingomyelinase, which has been demonstrated to have substantial phospholipase C (PLC) activity (Hanada 2002). The enzyme may be needed for degradation of host cell SM to supply ceramide to the parasite, which could adjust its cell cycle progression and/or could be used for SM re-synthesis within the parasite. Parasite-specific glycolipids were identified following metabolic labeling with tritiated serine and glucosamine and characterized as sphingolipids. The *de novo* synthesis of parasite glycosphingolipids was affected by fumonisin B1, threo-PPMP (phenyl-2-palmitoylamino-3-morpholino-1-propanol), cycloserine, and myriocin (well-established inhibitors of *de novo* ceramide biosynthesis). However, they were unable to arrest the intraerythrocytic development of the parasites in culture (Gerold and Schwarz 2001).

Isoprenoid

Isoprenoid is an organic compound composed of two or more units of hydrocarbons, with each unit consisting of five carbon atoms (isoprene) arranged in a specific pattern. Steroids, cholesterol, retinoids, carotenoids, ubiquinones, and prenyl groups bound to proteins are essential components in many types of cells and participate in various cellular functions. In malaria parasites they are synthesized by the methylerythritol phosphate pathway (MEP), which is located in the apicoplast. Using metabolic labeling with [1-^{14}C]acetate, D-[U-^{14}C]glucose and [2-^{14}C]1-deoxy-D-xylulose 5-phosphate (DOXP), the functionally active pathway was shown to be present in the erythrocytic stages of *P. falciparum* (Cassera 2004). All enzymes shown in Figure 9.11 are encoded by the nuclear genome and have atypical bipartite signal sequence that targets expressed proteins to the apicoplast. All intermediates of the pathway were identified, including those that serve the synthesis of ubiquinone and dolichol, as well as the expected inhibition by fosmidomycin.

DOXP synthase (EC 2.2.1.7), DOXP reductoisomerase (EC 1.1.1.267), and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (EC 4.6.1.12) have been characterized biochemically (Grawert 2011). DOXP reductoisomerase was shown to be essential for intraerythrocytic development of *P. falciparum* (Odom and Van Voorhis 2010). An initial ultrastructual analysis of DOXP reductoisomerase has also been reported (Umeda 2010). Being distinct from the mevalonate pathway of isoprenoid synthesis in vertebrates, the DOXP pathway has been the subject of targeted drug development (Jordao 2011).

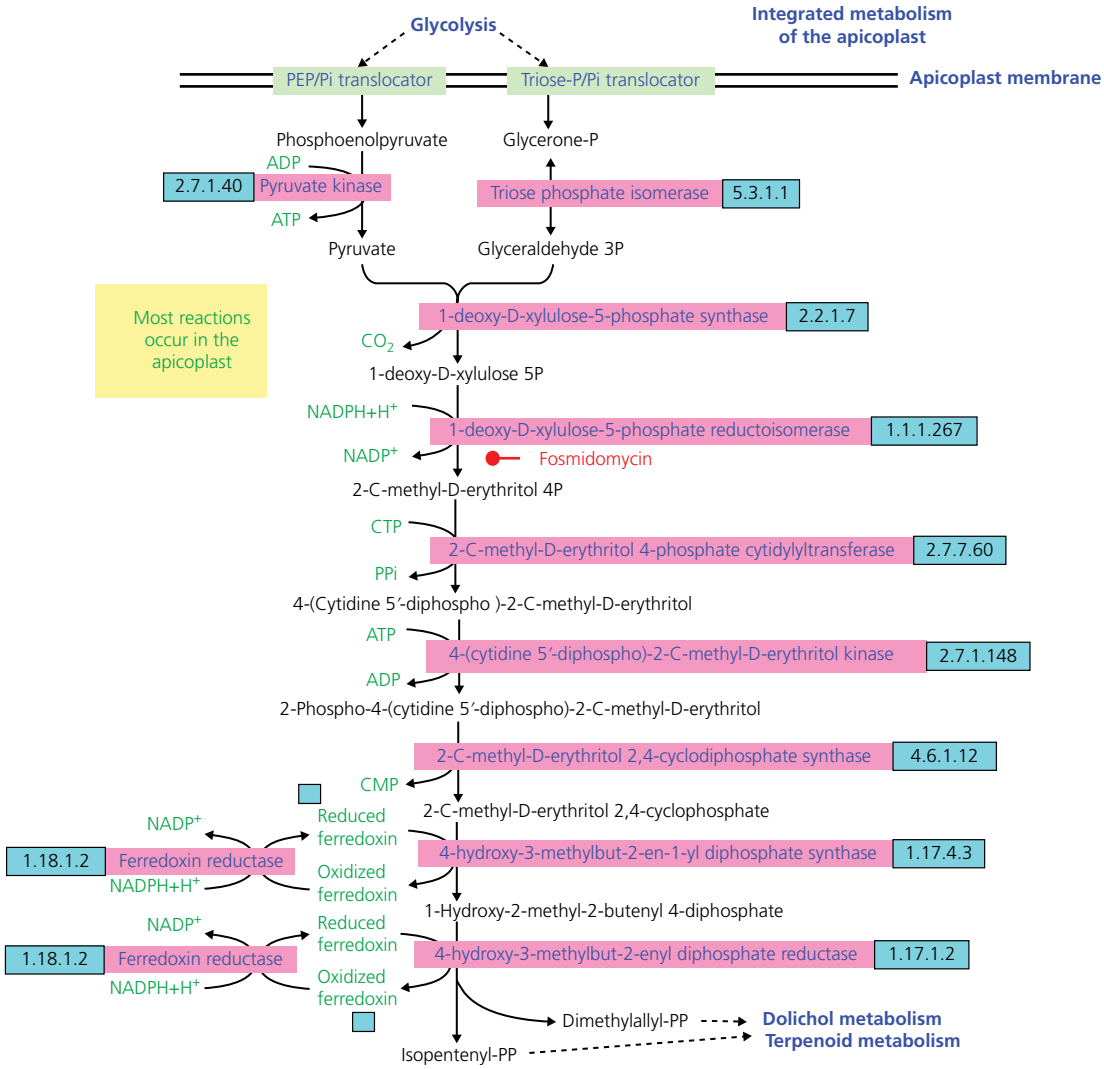


Figure 9.11 Isoprenoids metabolism. This pathway derives its substrates from glycolysis and provides substrates for the synthesis of terpenoids (Figure 9.12). It resembles the plant biosynthetic pathway and occurs in the apicoplast. Of note is the use of ferredoxin for redox cycling of NADP/H.

Terpenoid

The final products of isoprenoid metabolism are isopentenyl pyrophosphate and dimethylallyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are all intermediates of synthesis of a large variety of end products. GPP and GPPP serve as substrates for ubiquinone and dolichol generation. Using [^{3}H]FPP and [^{3}H]GGPP, it was possible to identify dolichol, dolichyl phosphate and dolichyl pyrophosphate of 55 and 60 carbons (11 and 12 isoprene units) and N-linked glycoproteins (Couto 1999). FPP and GGPP are also involved in post-translational modifications of proteins. Prenylated proteins labeled with [^{3}H]farnesol are mostly observed in

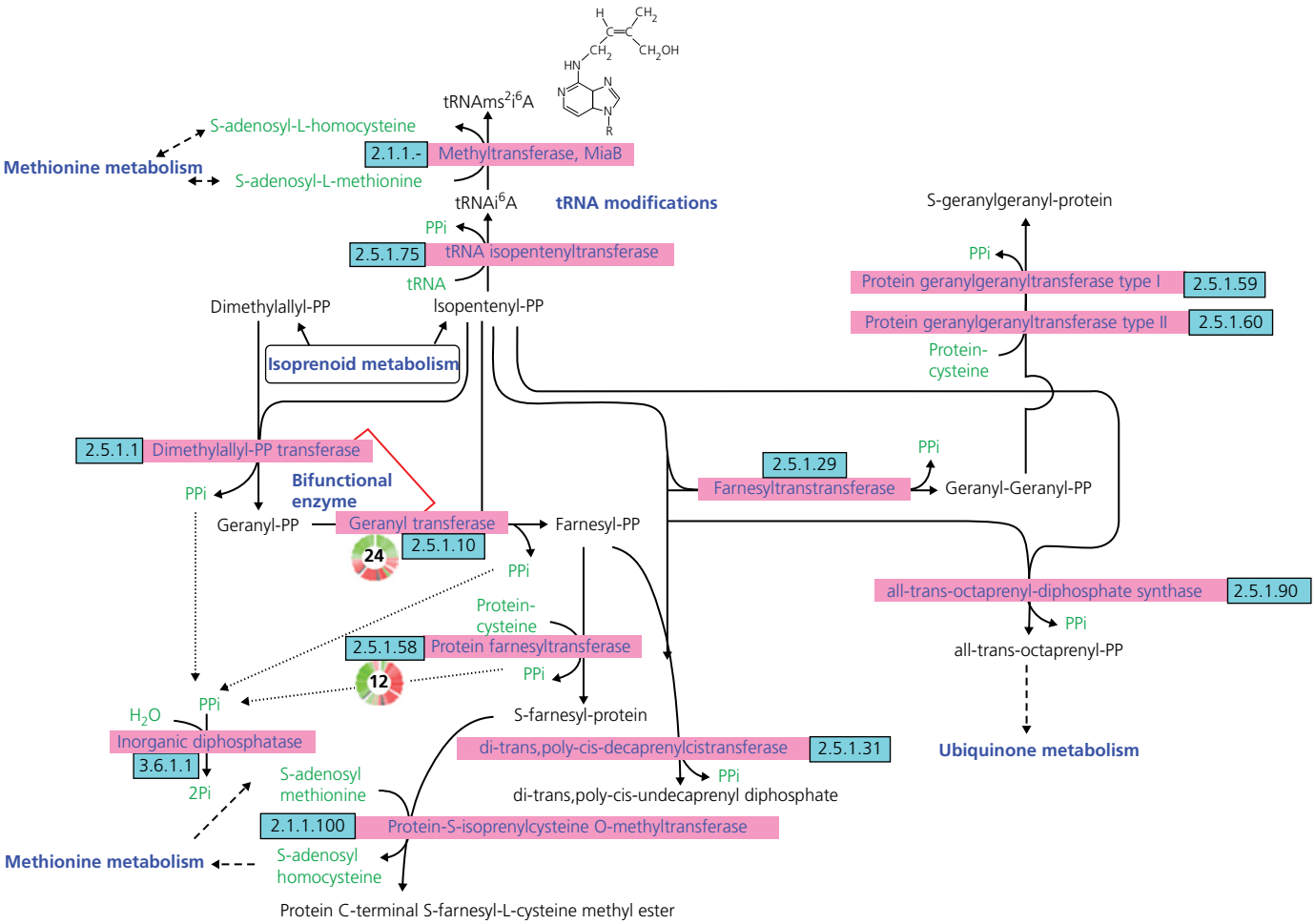


Figure 9.12 Terpenoids metabolism. Using dimethylallyl-PP and isopentenyl-PP, the pathway produces farnesyl, geranyl, and geranylgeranyl moieties for the posttranslational modification of proteins. Geranylgeranyl can be further elongated by adding prenyl (3-methyl-but-2-en-1-yl) moieties.

both 50- and 22- to 28- kDa ranges during the trophozoite to schizont stages, whereas fewer prenylated protein are observed in the ring to trophozoite stages (Chakrabarti 2002), suggesting that these post-translational modifications are significant for parasite differentiation. Prenyl transferase activity (EC 2.5.1.58, 2.5.1.59, 2.5.1.60) has been detected only in the cytosolic fractions (Chakrabarti 1998), from where it has been partially purified. Additional parasite enzymes involved in further modifications of FPP and GGPP have also been identified *in silico* but have yet to be tested biochemically. Several inhibitors against enzymes of terpenoid protein modification have also been tested successfully (Jordao 2011).

Dolichol

Probably the most important role of dolichol metabolism is to generate phosphorylated dolichols that function as carriers of oligosaccharides in the biosynthesis of N-linked glycoproteins and glycosylphosphatidyl inositol anchors. The synthesis of dolichol itself depends on the supply of FPP by the isoprenoid metabolic pathway (Cassera 2004). Different terpenes inhibited dolichol biosynthesis when [^{3}H]FPP was used as a precursor (Rodrigues 2004).

As shown in Figure 9.13, the generation of dolichyl phosphate D-mannose that functions in GPI anchor synthesis involves a close cooperation with the mannose metabolic pathway. Dolichol phosphate can also be generated by biochemically characterized activity of dolichol kinase (EC 2.7.1.108) by adding extrinsic dolichol and CTP, the phosphate donor provided by pyrimidine metabolic pathway (Walter 1986). The fact that this reaction must be provided from an extracellular source other than the parasite is suggested by the absence of the dolichyl-phosphatase encoding gene from the *P. falciparum* genome. On the other hand, production of the typical chloroplast lipids

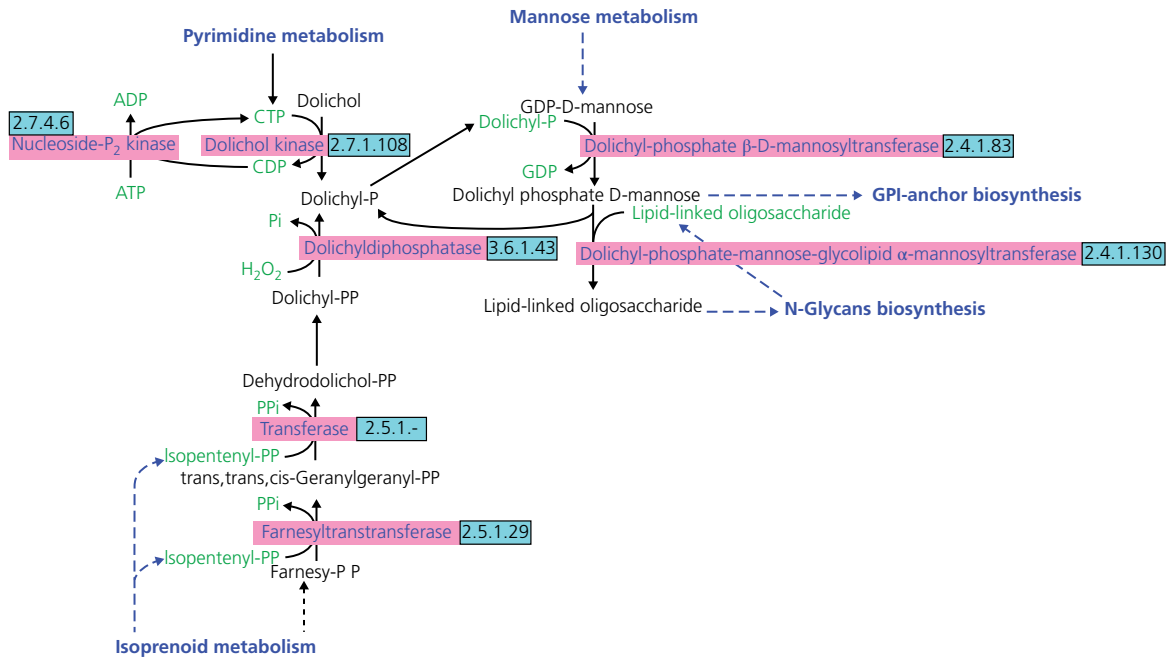


Figure 9.13 Dolichol metabolism. Dolichol cycles in the endoplasmic reticulum (ER), where the dolichol phosphate pool provides for *N*-glycosylation, *O*-mannosylation and GPI-anchor biosynthesis. The substrates for dolichol biosynthesis are produced in the apicoplast and must be transferred to the ER.

monogalactosyldiacylglycerol and digalactosyldiacylglycerol could be generated using *P. falciparum* lysate and UDP-[^{3}H] galactose. The radioactive label was found to be incorporated into monogalactosylcerebrosides, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol (Marechal 2002).

Glycosylphosphatidylinositols (GPIs)

Glycosylphosphatidylinositol (GPI) anchoring of proteins is a conserved post-translational modification in eukaryotes. GPI is synthesized and transferred to proteins in the endoplasmic reticulum. GPI-anchored proteins are then transported from the endoplasmic reticulum to the plasma membrane through the Golgi apparatus. GPI anchor is the main carbohydrate modification in parasite proteins, and GPIs are essential for parasite survival. GPI biosynthesis (Figure 9.14) predominates all other glycosylation activities in the parasite. The effects of parasite GPI anchors and glycolipids during *P falciparum* infection are beyond the scope of this review chapter and are summarized elsewhere (Schofield and Hackett 1993). GPI synthesis comprises a sequence of steps that involve inositol acylation and the consecutive transfer of various sugars from activated sugar donors and ethanolamine to the glycan core of these glycolipids. The incorporation of ethanolamine is distinctive to the *P. falciparum* GPI core-glycan-structure enterprise and is the final requirement for attachment of GPI to the C-terminus of proteins, which anchors them into the lipid bilayers and eventually transports the conjugate to the cell surface.

The pathway involves reactions occurring on both sides of the ER membrane. Two immediate GPI precursors with structures ethanolamine phosphate-6(Manα1-2)Manα1-2Manα1-6Manα1-4GlcN-PI and ethanolamine phosphate-6Manα1-2Manα1-6Man-α1-4-GlcN-PI are synthesized by *P. falciparum* (Gerold 1994; Debierre-Grockiego and Schwarz 2010). Several merozoite proteins involved in erythrocyte invasion are GPI anchored, and they either decorate the merozoite surface or reside in secretory organelles. Systematic attempts to disrupt the genes encoding seven of the known GPI-anchored merozoite proteins revealed that most of the genes (six of seven) are refractory to genetic deletion, with the exception being the gene encoding merozoite surface protein 5, MSP-5 (Sanders 2006). The molecular details of these MSP proteins has been discussed in great detail previously (von Itzstein 2008) as well as in Chapter 3 of this book.

Amino acids

The parasite digests up to 65% of its host cell cytosol, which consists mainly of Hb, but it uses only about 15% of the resulting amino acids for protein biosynthesis (Krugliak 2002). This result does not contradict the observation that parasite growth in culture depends on the presence of a minimal set of amino acids besides isoleucine, which must be obtained from an external source because it is absent in human Hb.

Amino acids exit the infected erythrocyte quite rapidly (Zarchin 1986). The rate of efflux depends on the concentration gradient and on the nature of the transport system at the parasite plasma membrane. The uptake of isoleucine is mediated by a leucine-dependent antiporter (Martin and Kirk 2007), and further interpretation of methionine transport implies that the parasite cell membrane has one or more carriers that mediate the flux of a wide range of neutral amino acids (Cobbold 2011). The relative transport of each amino acid depends on the concentration gradient and its affinity for the carrier, which could sometimes lead to intracellular depletion of the amino acid, leading to a dependence on extracellular supplies.

A major question that arises is why the parasite is investing so many resources on Hb digestion when it is using only a small fraction of the resulting Hb amino acids. The resolution of this enigma was enabled by the development of an integrated mathematical model of homeostasis for an infected

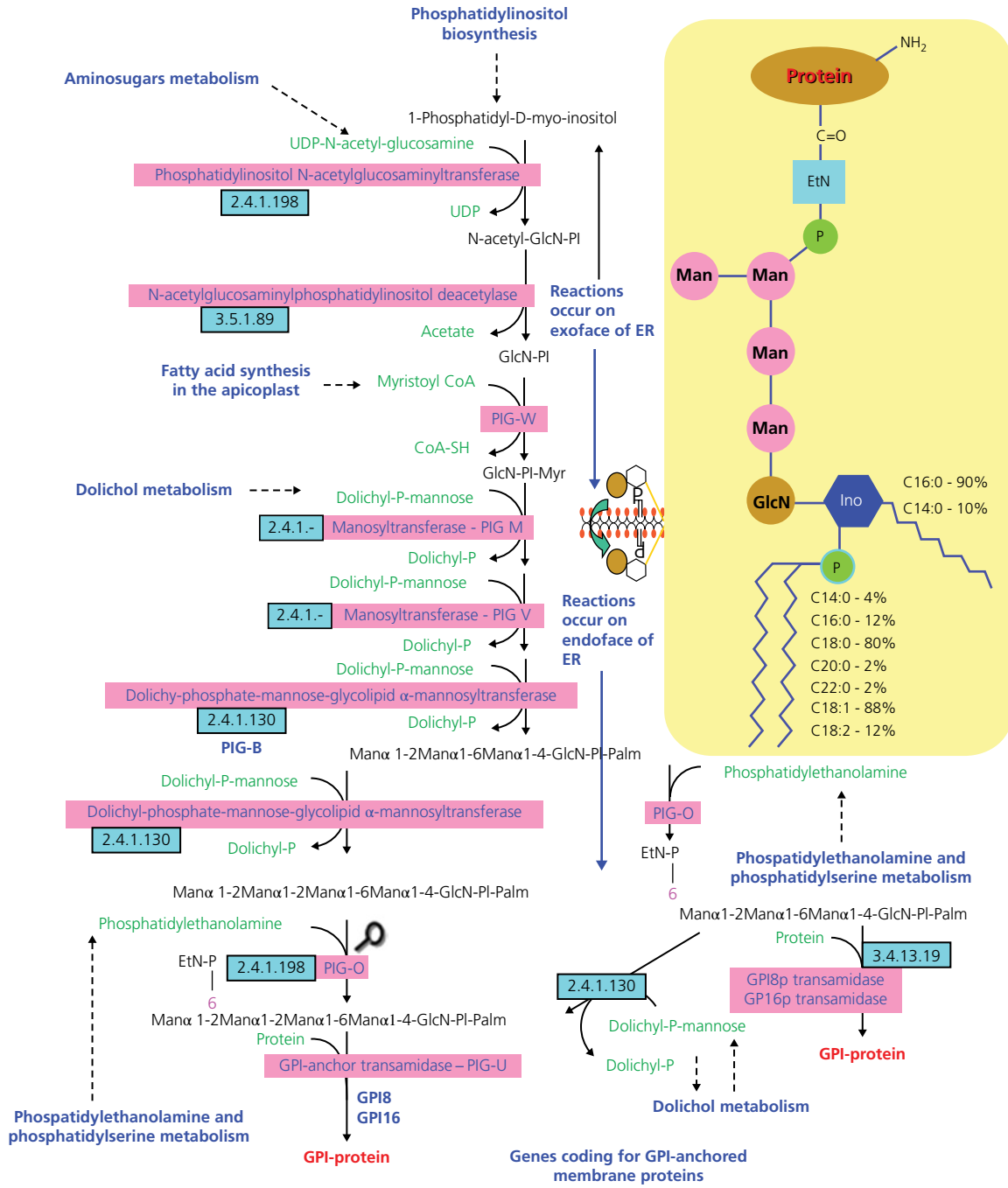


Figure 9.14 Biosynthesis of GPI anchors. GPI anchors serve to anchor proteins to membranes. Their structure, shown in the right upper corner, reveals that it is composed of inositol phosphate, sugars, and ethanolamine. The different lipid chains and their prevalence are also shown. Notice the first biosynthetic reactions occur at the exoface of the ER, and afterward GlcN-PI-Palm flips to endoface, where the path is completed by building the sugar tree.

erythrocyte. Model simulations revealed that excess hemoglobin digestion is needed for reducing the colloid–osmotic pressure within the host erythrocyte essential to preserve the osmotic stability of the infected cell during the parasite's asexual life cycle (Lew 2004). Although it seems that most amino acids exit the infected erythrocyte, some of them are metabolized within the infected cell to support important metabolic processes within the parasite.

Methionine and polyamine metabolism

Methionine metabolism plays a pivotal role in the parasite's physiology as a supplier of methyl moieties for methylation and for generation of polyamines (Figure 9.15). Methionine is converted by the action of methionine adenosyl transferase (EC 2.5.1.6) to *S*-adenosylmethionine, which donates methyl moieties through the action of various *S*-adenosyl-methyltransferases (Figure 9.14). The resulting *S*-adenosylhomocysteine is recycled back to methionine using adenosylhomocysteinase (EC 3.3.1.1) and homocysteine *S*-methyltransferase (EC 2.1.1.10).

Another important role of methionine metabolism is generation of polyamines. Polyamines are low-molecular-weight nitrogenous bases that are essential for enabling well-regulated cell growth and development. Being polycations, putrescine, spermidine, and spermine interact electrostatically with majority of polyanioninc macromolecules in cells, affecting different biological processes such as cell differentiation and proliferation.

Increased levels of polyamines and their biosynthetic enzymes are observed in highly proliferating cells, including parasites (Clark 2010). Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase usually regulate polyamine metabolism. Polyamine synthesis is supplied from *S*-adenosylmethioninamine, which is formed from *S*-adenosylmethionine by means of adenosylmethionine decarboxylase (EC 4.1.1.50), together with putrescine formed from ornithine by means of ornithine decarboxylase (EC 4.1.1.17). *S*-adenosylmethioninamine is then converted to spermidine by spermidine synthase (EC 2.5.1.16). No spermine synthase activity has been found, but low levels of spermine were produced by spermidine synthase (Haider 2005).

Interestingly, the two rate-limiting decarboxylases, adenosylmethionine decarboxylase and ornithine decarboxylase, are found in a bifunctional arrangement in a single protein complex (Müller 2000), and their coordinated expression has been suggested to control polyamine metabolism (Wrenger 2001). Polyamines can reach a concentration of 10 mM (spermidine 6 mM, putrescine 3 mM, and spermine 0.5 mM) inside the parasite and thus constitute about 14% of all parasite metabolites (Teng 2009; Olszewski 2009).

An inhibition of ornithine decarboxylase causes a cytostatic effect on parasite growth, which can be reversed by the addition of exogenous polyamines (Assaraf 1987). Morphological analysis of the 3D7 strain inhibited by cyclohexylamine, a spermidine synthase inhibitor, showed a full arrest of parasite development at the early trophozoite stage. In parallel, a transcriptional and translational arrest was observed for genes involved in polyamine biosynthesis as well as associated metabolic pathways that encode enzymes such as uridine phosphorylase, adenosine deaminase, lysine decarboxylase, *S*-adenosylmethionine synthetase, various methyltransferases, and enzymes involved in purine metabolism. These effects were reflected by changes in the levels of polyamines within the parasite (Becker 2010).

Spermidine is the substrate for a very specific post-translational modification of the translation initiation factor 5A to form the hypusine derivative, which is essential for cell proliferation (Park1997). Polyamine metabolism seems to be tightly connected with the redox metabolism as *S*-adenosyl homocysteinase binds to thioredoxin and glutaredoxin (Sturm 2009). Depletion of polyamines by inhibiting ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase caused a marked decrease in 1-Cys peroxiredoxin, thioredoxin, glutathione-*S*-transferase, and glutathione reductase (van Brummelen 2009). The merits of using components of polyamine biosynthesis as drug targets have been reviewed (Birkholtz 2011).

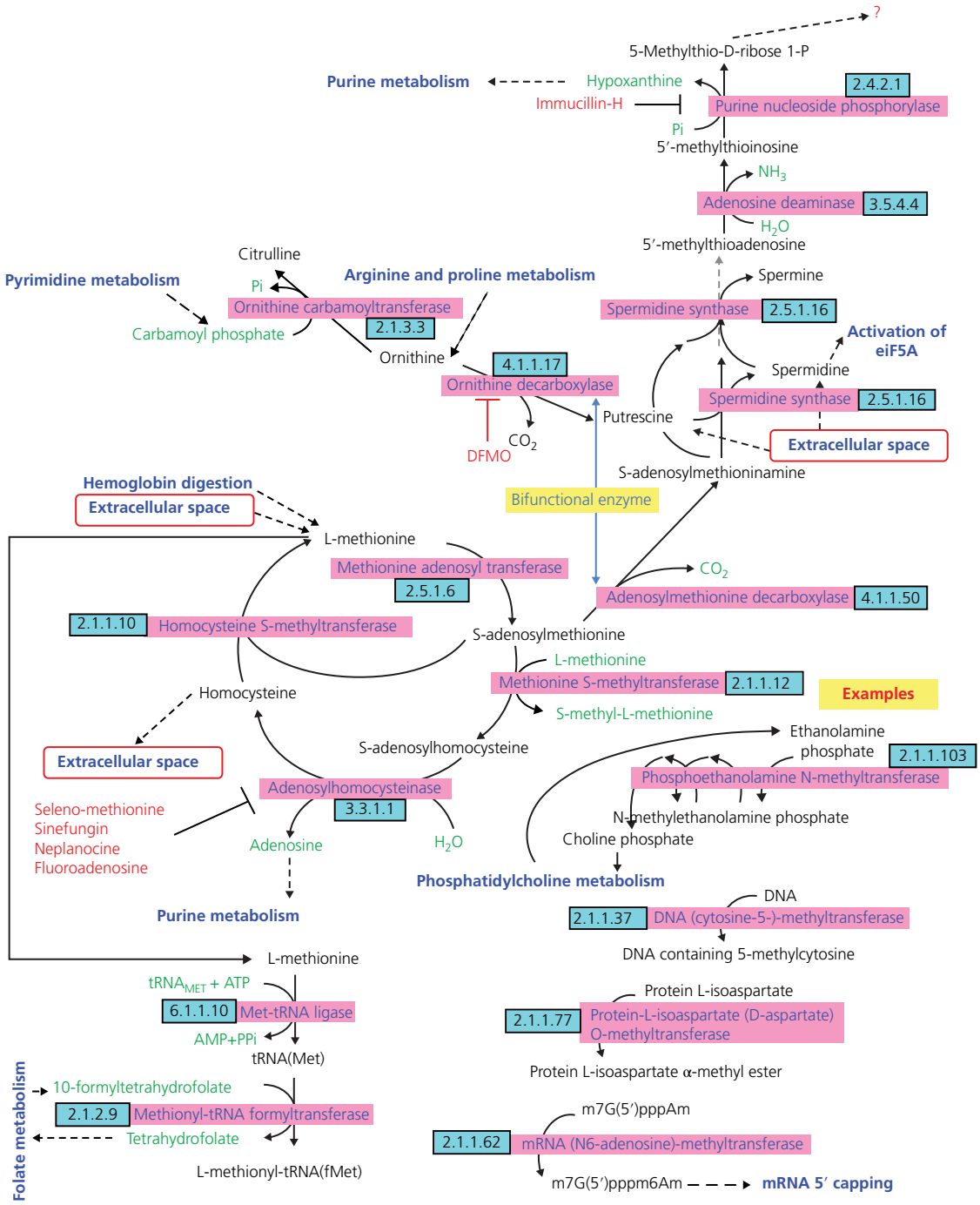


Figure 9.15 Methionine and polyamine metabolism. Methionine is one of the central amino acids in parasite metabolism. It provides methyl groups for the methylation of DNA, RNA, phospholipids, and proteins, mostly histones, and thus it is essential for regulation of gene expression. Polyamines are cationic compounds that play roles in the cell cycle, cell division, and differentiation.

Arginine and proline metabolism

The various metabolic fates of arginine and proline are shown in Figure 9.16A. Arginine is converted by arginase (EC 3.5.3.1) to ornithine, a substrate for polyamine synthesis. However, it seems that most of the proline is expelled from the infected erythrocyte (Olszewski 2009). Infected erythrocytes can efficiently deplete arginine from the culture medium. Arginine is a substrate for nitric oxide (NO) production, which is known to downregulate aggregation and adhesion and further decrease cytokine production, leading to reduced cytoadherence of infected erythrocytes to the microvascular endothelium (Serirom 2003). Thus, prevention of arginine efflux from the infected cell by its conversion to ornithine may be beneficial for parasite survival and propagation. Depletion of intracellular arginine could reduce NO production by the host cell, which could benefit the parasite as well. In fact, it has been suggested that replenishment of arginine and restoration of nitric

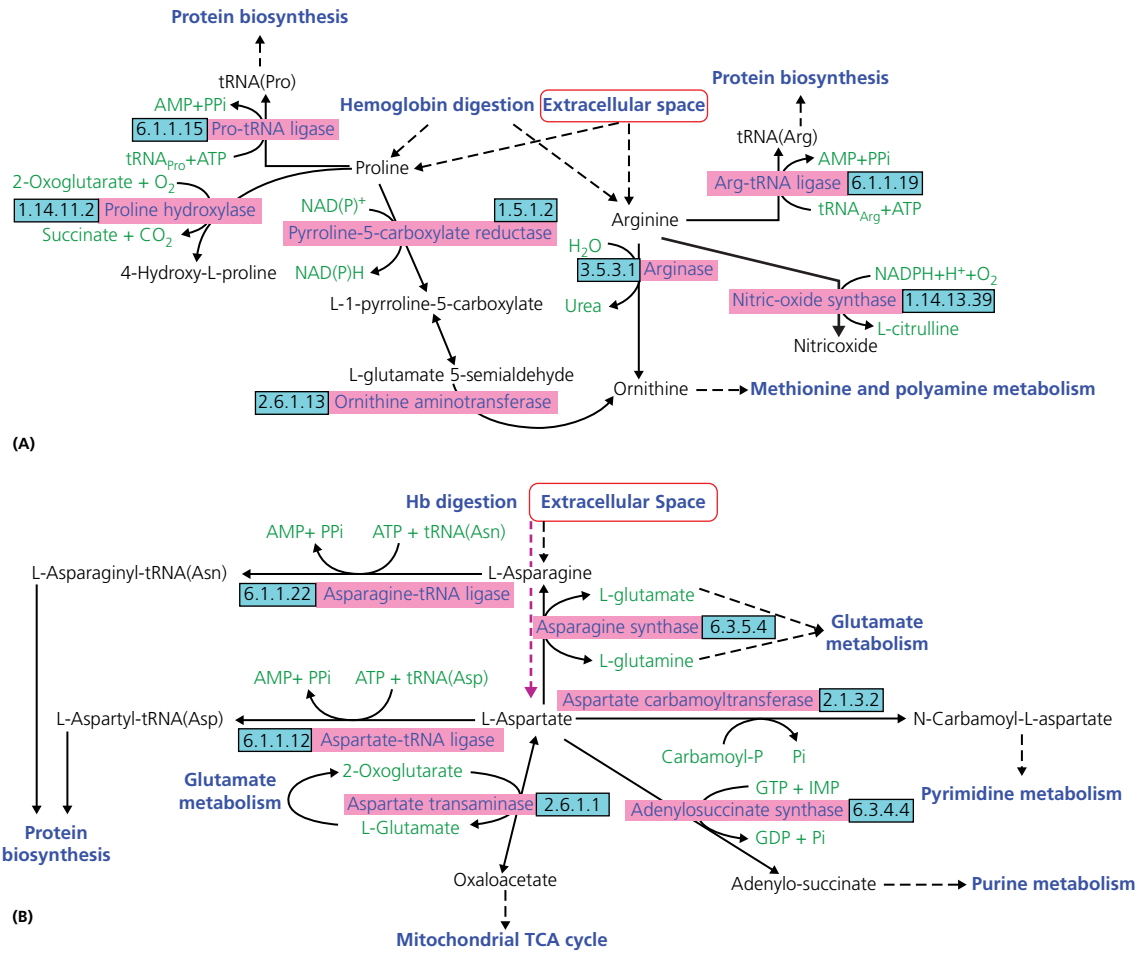


Figure 9.16 Arginine:proline:asparagine and aspartate metabolism. *A*, Arginine and proline provide ornithine for polyamine biosynthesis. The conversion of arginine to ornithine also reduces the levels of arginine, which is a substrate for NO synthesis, a noxious compound for the parasite. *B*, Aspartate and asparagine obtained from Hb digestion and uptake for the extracellular medium can interconvert. Aspartate is a major substrate for the generation of *N*-carbamoyl-L-aspartate, which in turn is a major substrate for pyrimidine synthesis and of adenylo-succinate, an intermediate in purine metabolism.

oxide production in clinical malaria should diminish the adherence of parasite-infected erythrocytes to the endothelium, which in turn would reduce the sequelae of these interactions (*e.g.*, cerebral malaria), and it argues for arginine therapy in addition to conventional antimalarials (Weinberg 2008). The kinetic properties of arginase have been characterized, and its three-dimensional crystal structure has also been reported at a resolution of 2.15 Å (Dowling 2010). The enzyme molecules have been reported to form Mn^{2+}-dependent trimers, which is obligatory for enzymatic activity (Wells 2009).

Proline is converted to L-1-pyrroline-5-carboxylate by pyrroline-5-carboxylate reductase (EC 1.5.1.2), and then it spontaneously forms L-glutamate 5-semialdehyde, which is converted by ornithine aminotransferase (EC 2.6.1.13) to ornithine, thus serving as an alternative for arginase, but this has yet not been demonstrated experimentally. The crystal structure of ornithine aminotransferase reported at a resolution of 2.3 Å has shown that the loop involved in substrate binding contains two cysteine residues, which were shown to mediate the interaction with thioredoxin. A firm thioredoxin-mediated control of ornithine aminotransferase activity may be involved in the regulation of ornithine homeostasis and hence the synthesis of polyamines.

Proline hydroxylase (EC 1.14.11.2) produces 4-hydroxy-L-proline, a known substrate of collagen production, which is probably absent in the intraerythrocytic parasite. However, the enzymatic activity involves the consumption of O_2 and has been implicated in O_2-sensing in *Dictyostelium*, as well as in vertebrates and invertebrates (West 2006).

Aspartate and asparagine metabolism

The metabolism of aspartate and asparagine is shown in Figure 9.16B. Both amino acids can be imported from the extracellular space or supplied by Hb digestion and can be interconverted by asparagine synthase (EC 6.3.5.4). Aspartate carbamoyltransferase (EC 2.1.3.2) binds carbamoyl phosphate to form the initial substrate for pyrimidine biosynthesis. The same pathway also obtains adenylosuccinate, which is formed from aspartate and inosine monophosphate (IMP) by adenylosuccinate synthase (EC 6.3.4.4). Aspartate transaminase (aminotransferase) (EC 2.6.1.1) catalyzes the conversion of aspartate and α-ketoglutarate into oxaloacetate and glutamate, thus participating in energy metabolism and *de novo* pyrimidine biosynthesis. The crystal structure of the recombinant aspartate transaminase enzyme at a resolution of 2.8 Å shows a truncation of its divergent N-terminal residues that leads to a loss of activity owing to its participation in catalysis and in the stabilization of the native homodimer (Wrenger 2011). Asn, Phe, Trp, and Tyr are also substrates to the enzyme, which is demonstrably cytosolic. Oxaloacetate produced by *P. falciparum* aspartate aminotransferase (PfAspAT) is converted into malate by PfMDH. Malate is then imported into the mitochondria by the malate–oxaloacetate antiporter and used by mitochondrial malate:quinone oxidoreductase as a substrate to reduce ubiquinone, which will then enter the electron transport chain through the bc1 complex.

Glutamate and glutamine metabolism

Glutamate and glutamine are involved in more than eight metabolic pathways of the parasite, the first being an acceptor of amino residues and the second as a donor of these moieties. The two can interconvert by means of a glutamate-ammonia ligase (EC 6.3.1.2). As shown in Figure 9.17, glutamate dehydrogenases (GDHs) are enzymes that catalyze the reversible oxidative deamination of L-glutamate to form 2-oxoglutarate (α-ketoglutarate) and ammonia using NADP(H) (EC 1.4.1.4, GDH a/ b) or NAD(H) (EC 1.4.1.2, GDH c) as a cofactor. GDHb is probably located in the apicoplast. NADP(H) is also a cofactor for glutamate synthase (EC 1.4.1.13), which also interconverts glutamate and 2-oxoglutarate.

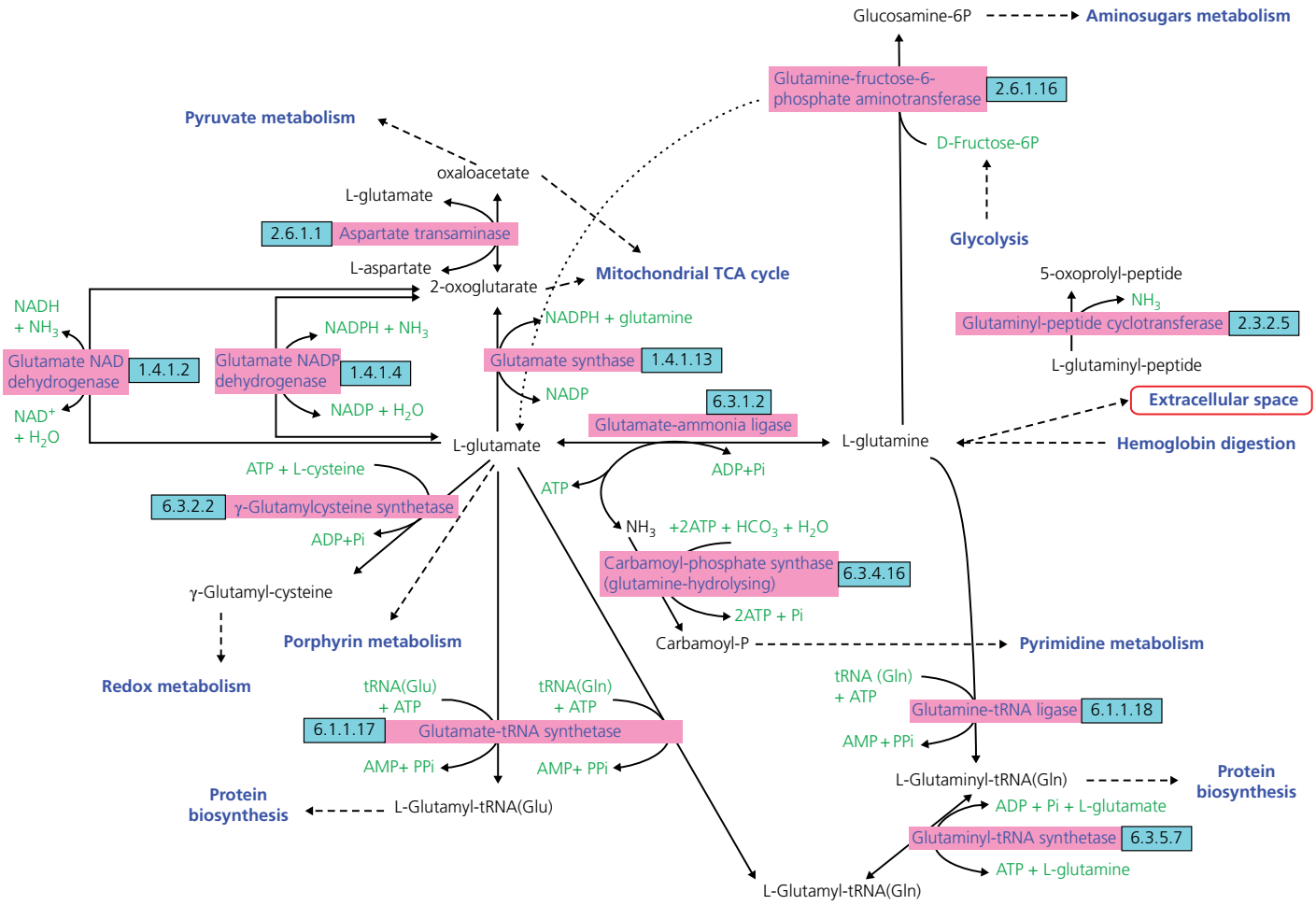


Figure 9.17 Glutamate and glutamine metabolism. While being obvious substrates for protein biosynthesis, glutamate serves as a substrate for the synthesis of glutathione and is also converted to α-ketoglutarate by three alternative paths, to be funneled to the TCA cycle. Glutamine, on the other hand, is a substrate for the synthesis of glucosamine-6-P and carbamoyl-P, which in themselves are substrates for aminosugar and pyrimidine biosyntheses, respectively.

Cytosolic GDH has been functionally characterized (Krauth-Siegel 1996) to show that it contains FAD as its prosthetic group and that it has much higher affinity to NAD than to NADPH, driving the reaction toward the production of 2-oxoglutarate. The *GDHa* gene has been cloned, and the *Escherichia coli*–expressed enzyme has been characterized biochemically. GDHa is present in all the erythrocytic stages, with increasing expression from the ring to late trophozoite stages (Wagner 1998). The structure of GDH has been determined to a resolution of 2.7 Å to show that the subunits are held together by salt bridges and a unique N-terminal extension not present in any other GDH (Werner 2005). The kinetic parameters suggest that the enzyme catalyzes both the reductive amination of α-ketoglutarate and the oxidative deamination of glutamate.

It has been suggested that a major role of GDHa is to provide reducing NADPH to the disulfide oxidoreductases: glutathione reductase and thioredoxin reductase (Müller 2003). Thus, GDH can be considered a legitimate member of the antioxidant defense of the parasite. GDHa null mutants were generated and their phenotypes were tested for their antioxidant resistance. No growth inhibition under different oxygen levels could be observed. Neither changed the levels of proteins involved in the generation of NADP(H) in the knockout strain. α-Ketoglutarate and other TCA intermediates were equally labeled with [^{13}C] in the wild-type and the mutant strains (Storm 2011).

It has been suggested that GDH is not important for the provision of NADP(H) for reductive reactions, but rather in ammonia assimilation (as a nitrogen storage molecule) in situations where the parasite faces a limited supply of carbon sources. However, this has yet to be validated *in vivo*.

Glycine and serine metabolism

Glycine and serine are obtained from the extracellular space and/or from Hb digestion. Serine is a source for the biosynthesis of phosphatidylserine. As shown in 9.18, serine can be transformed to glycine by serine hydroxymethyltransferase (SHMT, EC 2.1.2.1). Glycine can be converted to 5-aminolevulinate, a substrate of porphyrin biosynthesis by 5-aminolevulinate synthase (EC 2.3.1.37). Glycine is also the substrate for the mitochondrial glycine cleavage (GCV) system (Salcedo 2005). By acting in concert, these proteins catalyze the oxidative decarboxylation and deamination of glycine with the formation of CO_2, NH_3, and the concomitant reduction of NAD^+ to NADH. The remaining methylene carbon of glycine is then transferred to H_4folate (supplied by the folate pathway) to form CH_2H_4folate, which can then react with a second molecule of glycine in a reaction catalyzed by serine hydroxymethyltransferase to form serine. The folate derivatives are also needed for the biosynthesis of pyrimidines.

As shown in Figure 9.18, GCV is a loose assembly of four associated proteins: protein P (glycine dehydrogenase [decarboxylating], EC 1.4.4.2), protein T (aminomethyltransferase, EC 2.1.2.10), protein L (dihydrolipoyl dehydrogenase, EC 1.8.1.4), and protein H (PF11_0339). Protein H is not an enzyme, but it is modified with a lipoic acid co-factor that covalently binds with the reaction intermediates and shuttles them between the active sites of the enzymatic components of the complex. Glycine is decarboxylated by the P-protein and binds the methylamine group to the lipoic acid co-factor of protein H. A methylene group is transferred by the T-protein to H_4folate to form CH_2H_4folate. Lipoamide dehydrogenase reoxidizes dihydrolipoic acid to lipoic acid in a NAD^+-dependent reaction. Although the parasite contains cytosolic and mitochondrial SHMT isoforms (the latter was found to be inactive), GCV was found to be located in the mitochondrion (Spalding 2010).

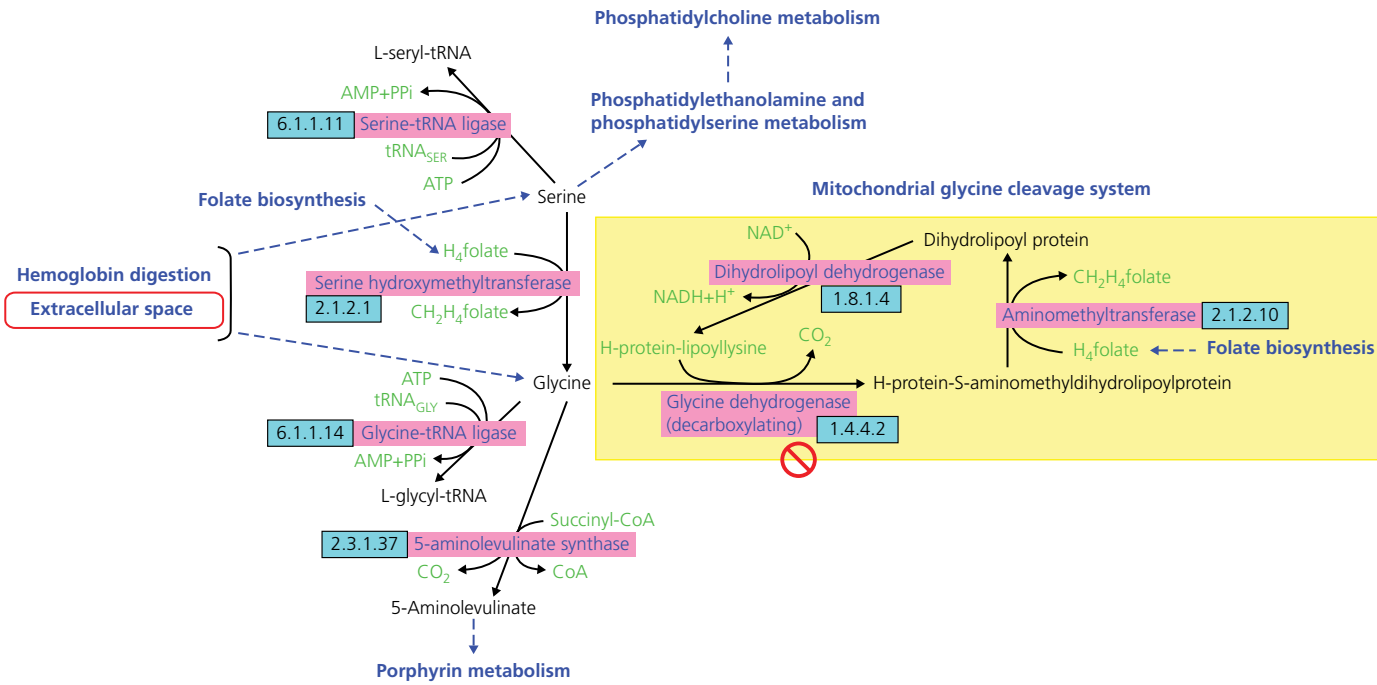


Figure 9.18 Glycine and serine metabolism.The glycine cleavage complex (GCV) is a source of the one carbon donor 5,10-methylene-tetrahydrofolate (CH_2H_4folate), necessary for nucleotide synthesis (see Figure 9.31). However, a gene necessary for encoding glycine dehydrogenase could not be found in the genome. Glycine is also a substrate for the synthesis of 5-aminolevulinate. which is used for porphyrin biosynthesis. Serine is a substrate for phosphatidylserine and for sphingomyelinbiosyntheses.

Leucine: isoleucine and valine metabolism

Leucine and valine can be obtained from Hb degradation or from the extracellular space, but isoleucine must be imported because it is absent from the structure of globin. In fact, the parasite can grow in culture when isoleucine is the only amino acid in the medium (Liu 2006). The uptake of isoleucine in trophozoite-infected erythrocytes is about five-fold higher than in uninfected cells. Isoleucine translocates across the host cell membrane through the new permeability pathways induced by the parasite in the host cell membrane (Staines 2007). Isoleucine is then transported across the parasite membrane via a saturable ATP/$Na^{+/}H^{+}$-independent system, which has the capacity to mediate the influx of isoleucine in exchange for leucine (obtained from hemoglobin) followed by molecular trapping (Martin and Kirk 2007).

As shown in Figure 9.19, all three amino acids can be transaminated by branched-chain-amino-acid transaminase (EC 2.6.1.42) to the corresponding keto-acids, which are then reduced sequentially by 3-methyl-2-oxobutanoate dehydrogenase (EC 1.2.4.4) and dihydrolipoyl dehydrogenase (EC 1.8.1.4). Next, dihydrolipoyl transacylase (EC 2.3.1.16) generates the respective acyl-CoAs. The following step is mediated by Acyl-CoA dehydrogenase (EC 1.3.1.8), whose encoding gene is missing from the genome. Enoyl-CoA hydratase (EC 4.2.1.17) and 3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4) are the last steps in the degradation of these amino acids, for which encoding genes could be identified. It should be noted that there are no evident metabolic processes in which the keto-acids and the acyl CoAs could be used, and there is no experimental evidence for the degradation of these amino acids.

Phenylalanine and tyrosine metabolism

Phenylalanine and tyrosine are obtained from either Hb digestion or an extrinsic source. As shown in Figure 9.20A), both amino acids can be partially degraded by the relatively nonspecific aspartate transaminase (EC 2.6.1.1) and aromatic-amino-acid transaminase (EC 2.6.1.57) to form phenylpyruvate and 4-hydroxy-phenylpyruvate, respectively, which can be further isomerized to 2-hydroxy-3-phenylpropenoate and 2-hydroxy-3-(4-hydroxy-phenyl)propenoate, respectively, by phenylpyruvate tautomerase (5.3.2.1). There is no experimental evidence that such degradation takes place, and it seems that the use of 2-oxoglutarate as a co-substrate for all transaminase activities seems to compete with the supply of this substrate to the mitochondrial TCA cycle.

Lysine metabolism

Lysine, obtained from Hb digestion or from the extracellular space, serves protein synthesis. As shown in Figure 9.20B, it can be degraded either by lysine decarboxylase (EC 4.1.1.18) to cadaverine or by saccharopine dehydrogenase (EC 1.5.1.7) to saccharopine. Further degradation of cadaverine could lead to 5-oxopentanoate via some spontaneous transformations and 5-aminovalerate transaminase (EC 2.6.1.48). No genes encoding enzymes required for further degradation of saccharopine could be found in the genome. It should be emphasized that there is no experimental evidence to support the actual degradation of lysine. Lysine is reported to inhibit heme polymerization with a K_m of 219 mM (Uyen 2008).

Selenocysteine

Selenium gets inserted in the protein in response to a UGA codon as selenocysteine (Sec), which is essentially the 21st amino acid in the genetic code. The presence of selenocysteine-tRNA and a selenocysteine insertion sequence in *P. falciparum* has been reported (Mourier 2005). *In silico* search in the genome for evolutionarily conserved selenocysteine insertion sequence (SECIS) elements, which are RNA structures involved in Sec insertion, has identified four unique parasite

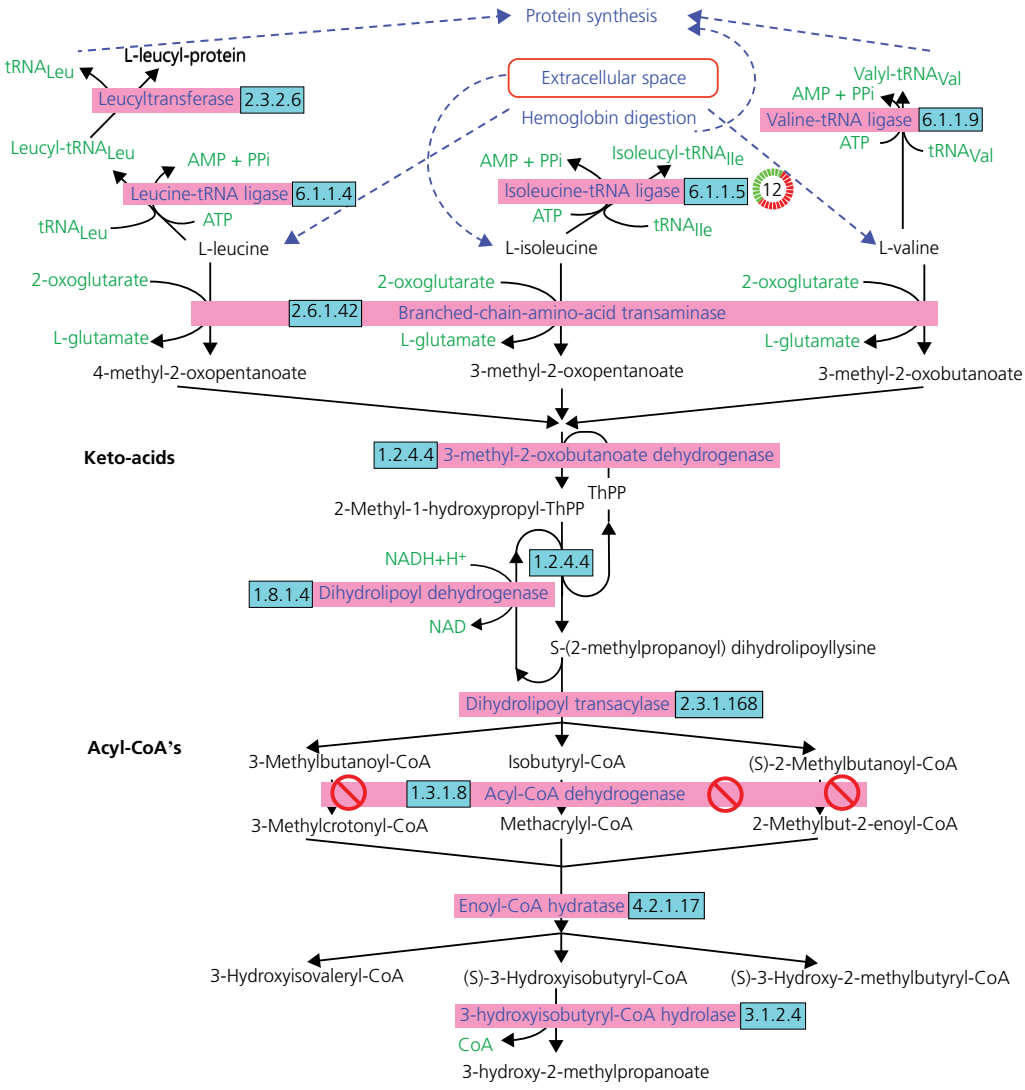


Figure 9.19 Leucine:isoleucine and valine metabolism. All three amino acids are possible subjected to degradation. However, the degradation pathways were constructed based on biochemical and genetic knowledge, but none of the degradation products has ever been recorded.

selenoprotein genes that have no orthologues in other species (Lobanov 2006) Two parasite SECIS elements were able to sustain Sec insertion into the parasite and endogenous selenoproteins when expressed in mammalian cells, thus indicating that the parasite SECIS elements are functional. All Sel genes were expressed throughout the erythrocytic stages. All known components of the Sec insertion machinery are present in the parasite, including tRNASec, SECp43/SLA/LP, L-seryl-tRNA(Sec) selenium transferase (EC 2.9.1.1). SECIS binding protein and selenocysteine-specific elongation factor have genes encoding for them in the genome (Figure 9.21). The transformation of selenite (imported from the extracellular space) to selenophosphate, the substrate of L-seryl-tRNA(Sec) selenium transferase, is mediated by selenide water dikinase (EC 2.7.9.3).

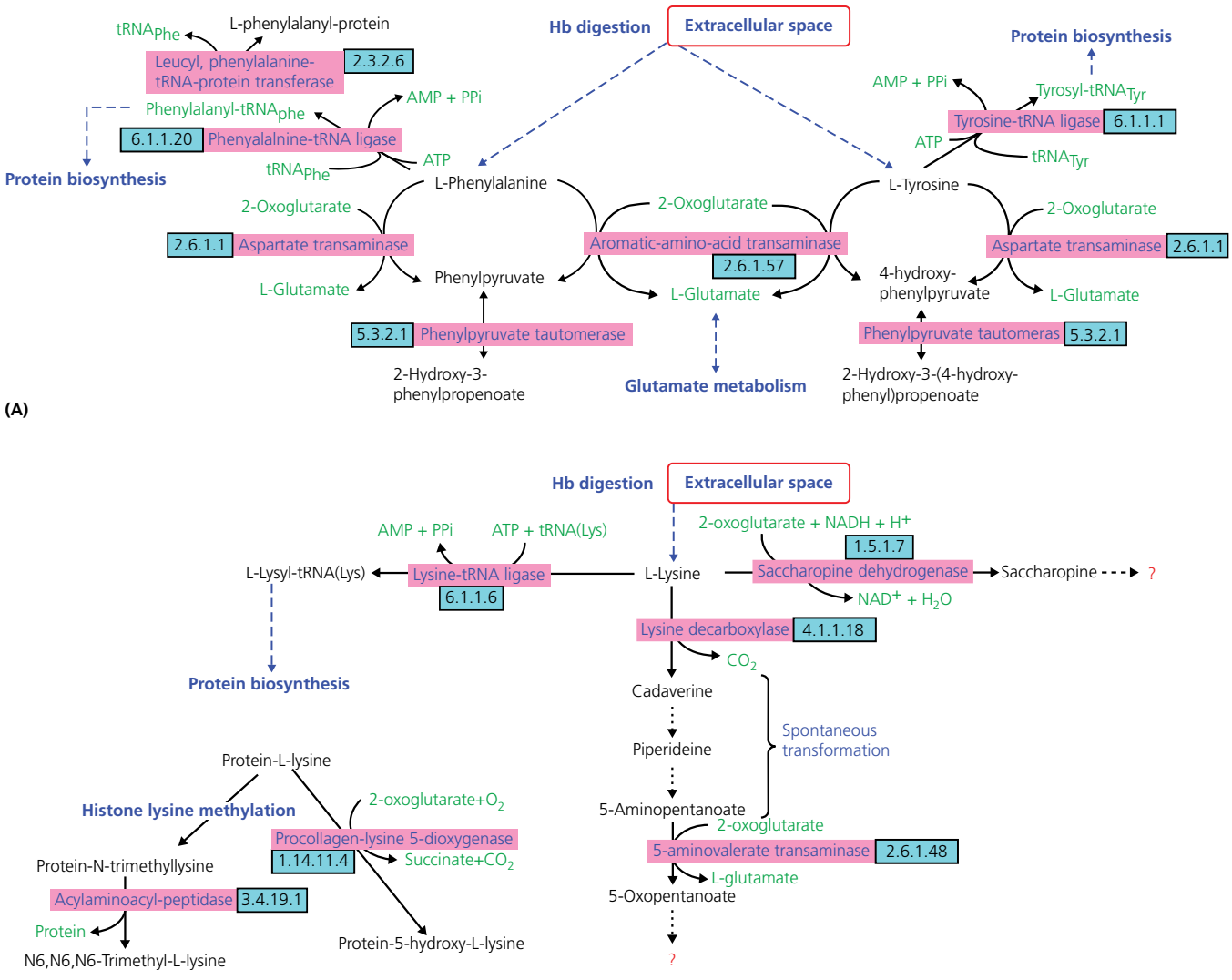
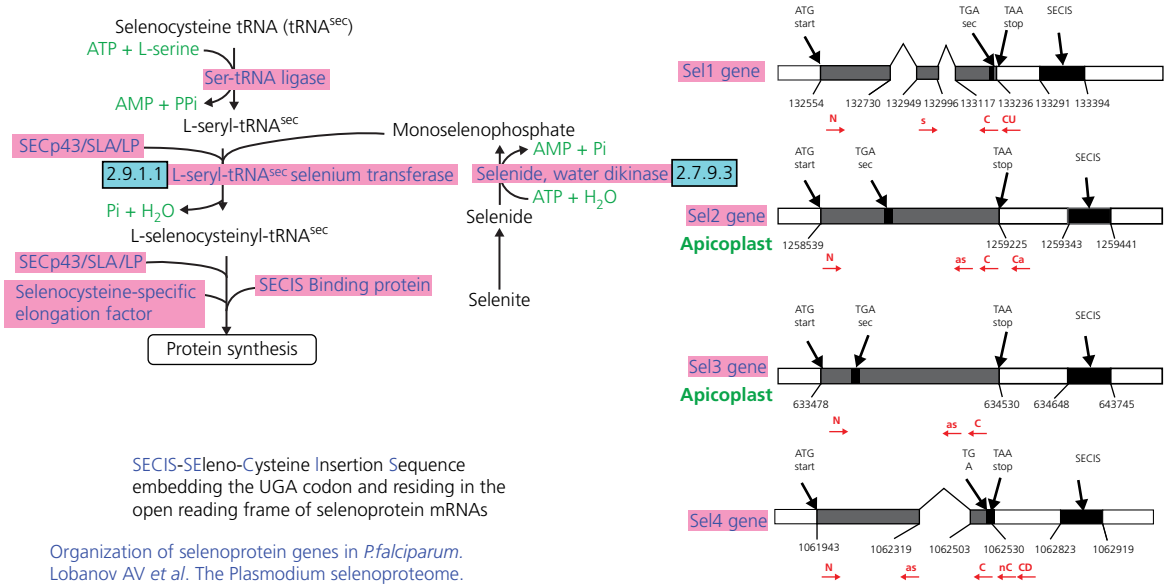


Figure 9.20 Phenylalanine-tyrosine and lysine metabolism. *A*, Phenylalanine and tyrosine can possibly be degraded by the concerted actions of aspartate transaminase, aromatic-amino-acid transaminase, and phenylpyruvate-tautomerase. These enzymes are not specific for the substrates shown here, but they have a rather wide range of substrates, allowing the drawing of this chart. None of the degradation products has ever been reported. B, Lysine degradation is mediated by rather specific enzymes for which encoding genes have been found in the genome. The possible roles of saccharopine and 5-oxopentanoate are not known and were not identified yet in the parasite.



SECIS-SElenoCysteine Insertion Sequence embedding the UGA codon and residing in the open reading frame of selenoprotein mRNAs

Organization of selenoprotein genes in *P.falciparum*. Lobanov AV *et al*. The Plasmodium selenoproteome. Nucleic Acids Res. 2006, 34:496–505

Exon-intron structure of the four selenoprotein genes is shown with exons indicated by boxes and introns by lines connecting the exons. The location of ORFs and SECIS elements is indicated by filled boxes, and the untranslated regions (other than SECIS elements) by open boxes.

Figure 9.21 Selenocysteine metabolism. The role of this 21st amino acid in parasite proteins has received little attention, but all the elements that are involved in its metabolism are present.

Nucleotide metabolism

Purine biosynthesis

Replication of DNA and its transcription to various RNA species require a constant and extensive supply of the component nucleotides for rapidly dividing organisms. *P. falciparum* is a purine auxotroph, salvaging host cell purines for synthesis of co-factors and nucleic acids. As shown in Figure 9.22, the parasite imports nucleobases and nucleosides from the host cell by means of PfNT1, a nucleoside transporter with broad substrate specificity for both purines and pyrimidines (Carter 2000; Parker 2000; Downie 2008). A second transporter, PfNT2, has been identified and located at the ER membrane, where its function is not evident (Downie 2010). Although hypoxanthine is considered as the preferred precursor (Sujay & Balaram 2000), all imported substrates are transported into the purine nucleotide pathway (Figure 9.21). Thereafter, using purine nucleoside phosphorylase (EC 2.4.2.1), adenosine deaminase (EC 3.5.4.4), and hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8), which incorporates phosphoribosyl pyrophosphate generated by the pentose phosphate pathway, the final product is inosine monophosphate (IMP). IMP is then converted to GMP and AMP by two parallel branches.

Guanosine monophosphate (GMP) and adenosine monophosphate (AMP) are then phosphorylated respectively by guanylate kinase (EC 2.7.4.8) and adenylate kinase (EC 2.7.4.3) to GDP and ADP. These are converted to their respective deoxynucleotides catalyzed by ribonucleotide diphosphate reductase (EC 1.17.4.1), where reduced thioredoxin (Trx) supplies the reducing power. The deoxynucleotides dGDP and dADP and the nucleotides GDP and ADP are then phosphorylated by nucleoside-diphosphate kinase (EC 2.7.4.6) to their respective trinucleotides, the first two serving DNA synthesis and the latter two RNA synthesis.

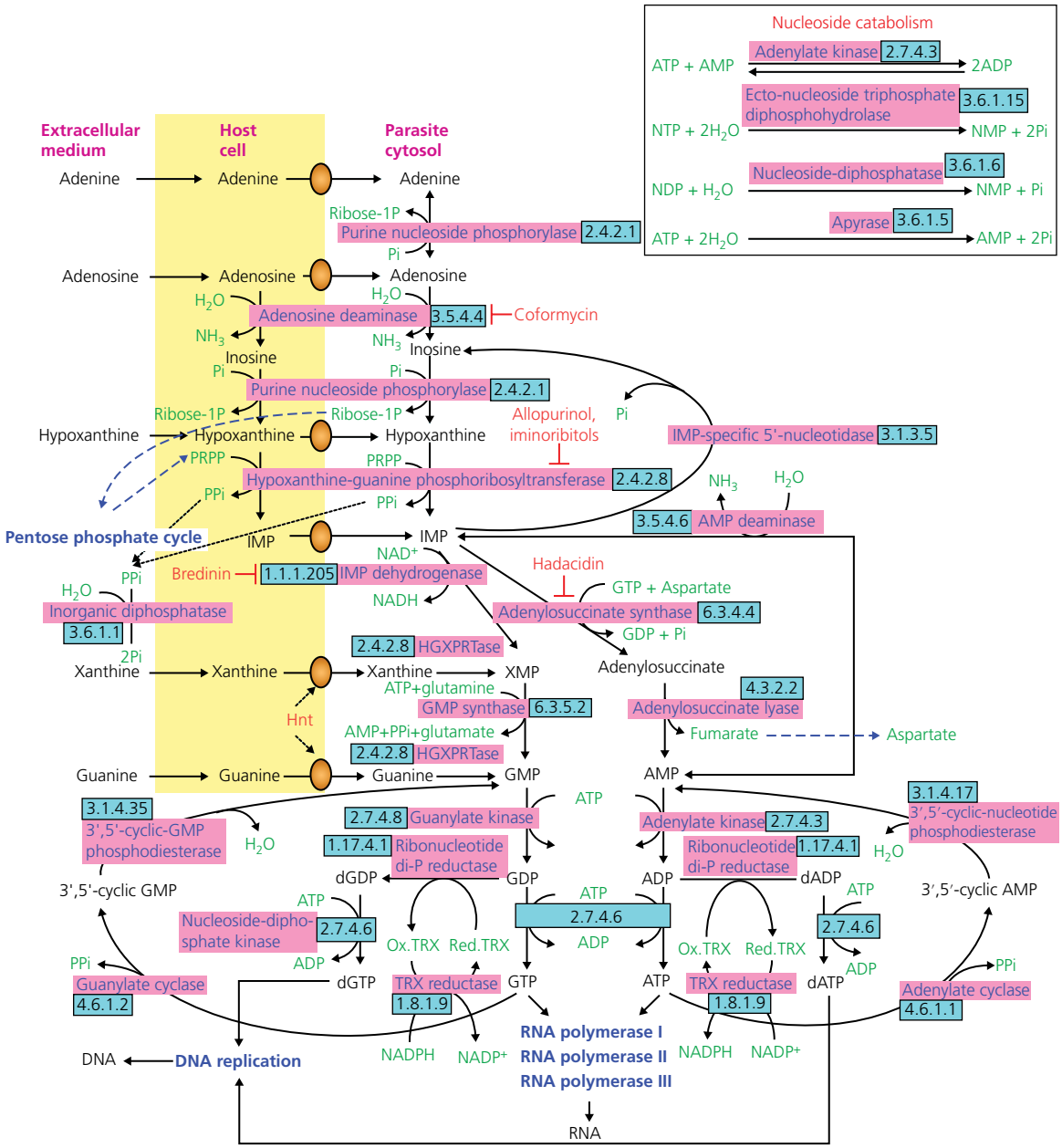


Figure 9.22 Purine biosynthesis. The parasite is a purine auxotroph, salvaging host cell purines, preferring hypoxanthine over other nucleobases and nucleosides. This preference is met by the extensive purine salvage processes that take place in the host cell, shown in yellow background. As shown, the starting points for purine biosynthesis may be at different points on the pathway with different substrates, provided all substrates can be imported by means of the nucleotide (Pfnt) carrier. At the end of the paths, deoxynucleosides are used for DNA synthesis and nucleosides for the various types of RNA. Also shown in the inset at the right upper corner are the various paths of nucleoside catabolism.

Evidence has been presented that implies adenosine kinase and adenine phosphoribosyltransferase in the production of AMP when hypoxanthine–guanine phosphoribosyltransferase is inactive although no genes have been annotated for these enzymes in the *P. falciparum* genome (Mehrotra 2010). The crystal structure and some biochemical properties of purine nucleoside phosphorylase have been investigated (Schnick 2005; Shi 2004). The crystal structure of the transition state of purine nucleoside phosphorylase with the inhibitor immucillin H and two Mg^{2+} ions has been resolved to show unique features of the transition-state analogue complex (Shi 1999). The biochemical and structural properties of GMP synthetase have been investigated to show it to be the glutamine-hydrolyzing type (EC 6.3.5.2) (Bhat 2008, 2011a, 2011b). Together with GMP synthetase, genes encoding adenoylsuccinate synthase (EC 6.3.4.4) and adenoylsuccinate lyase (EC 4.3.2.2) have been characterized (Marshall & Coppel 1997; Jayalakshmi 2002; Eaazhisai 2004; Bulusu 2009; Mehrotra 2012). Thus a complete set of enzymes is present to catalyze the interconversion of AMP, IMP, XMP, and GMP. Other enzymes encoded by the genome that are involved in the catabolism of purine nucleotides are shown in the inset in Figure 9.22.

One of the by-products of purine salvage is the fumarate produced by adenoylosuccinate lyase. It has been suggested that this cytosolic fumarate is translocated into the mitochondrion, where it is metabolized to malate fumarate hydratase (EC 4.2.1.2) and then converted to oxaloacetate by malate:quinone oxidoreductase (EC 1.1.99.16), which is finally re-translocated into the cytosol, where it is converted to aspartate by aspartate aminotransferase (Mehrotra 2010). This peculiar path was suggested to serve a role in replenishing the electron transport chain through the conversion of malate to oxaloacetate, thereby supporting the maintenance of the mitochondrial membrane potential needed for protein uptake.

Pyrimidine biosynthesis

P. falciparum depends on *de novo* biosynthesis for supply of pyrimidine nucleotides for DNA and RNA synthesis akin other eukaryotes. It starts from the amino acids glutamine and aspartic acid provided either by digestion of ingested Hb or by import from the extracellular space. As shown in Figure 9.23, glutamine and bicarbonate are used by carbamoyl-phosphate synthase (EC 6.3.5.5) to form carbamoyl phosphate, which then incorporates aspartate through the mediation of aspartate carbamoyltransferase (EC 2.1.3.2) to form *N*-carbamoyl-L-aspartate, which is further transformed into L-dihydroorotate by dihydroorotase (EC 3.5.2.3). The next enzyme is flavoenzyme dihydroorotate dehydrogenase (EC 1.3.98.1), which is anchored in the inner mitochondrial membrane and oxidizes L-dihydroorotate-dihydroorotate to orotate, releasing an electron to the electron transport chain. This enzyme has been isolated and characterized (Krungkrai J 1995), and it is receiving great attention as a drug target (Phillips & Rathod 2010).

Orotate phosphoribosyl transferase (EC 2.4.2.10) then incorporates ribose from phosphoribosyl pyrophosphate to generate orotidine-5′-phosphate. This is then decarboxylated by orotidine-5′-phosphate decarboxylase (EC 4.1.1.23) to uridine 5′-monophosphate (UMP), the precursor of all other pyrimidine nucleotides. Orotate phosphoribosyltransferase and orotidine 5′-monophosphate decarboxylase exist as multienzyme complexes, as in other eukaryotes (Krungkrai J 1995), although the genes have prokaryotic origin (Krungkrai 2005).

Two consecutive phosphorylations of UMP by cytidylate kinase (EC 2.7.4.14) and nucleoside–diphosphate kinase (EC 2.7.4.6) yield UTP, which is then converted to CTP by CTP synthase (EC 6.3.4.2). UTP and CTP are substrates for RNA synthesis. UDP and CDP are converted to their deoxy forms by ribonucleotide reductase (EC 1.17.4.1), which are subsequently phosphorylated to be channeled to DNA synthesis. The change of deoxyuridine 5′-monophosphate (dUMP) to deoxythymidine 5′-monophosphate (dTMP) involves folate cofactors (see the section Folate metabolism). For every molecule of dUMP, a molecule of 5,10-methylenetetrahydrofolate transfers its one-carbon

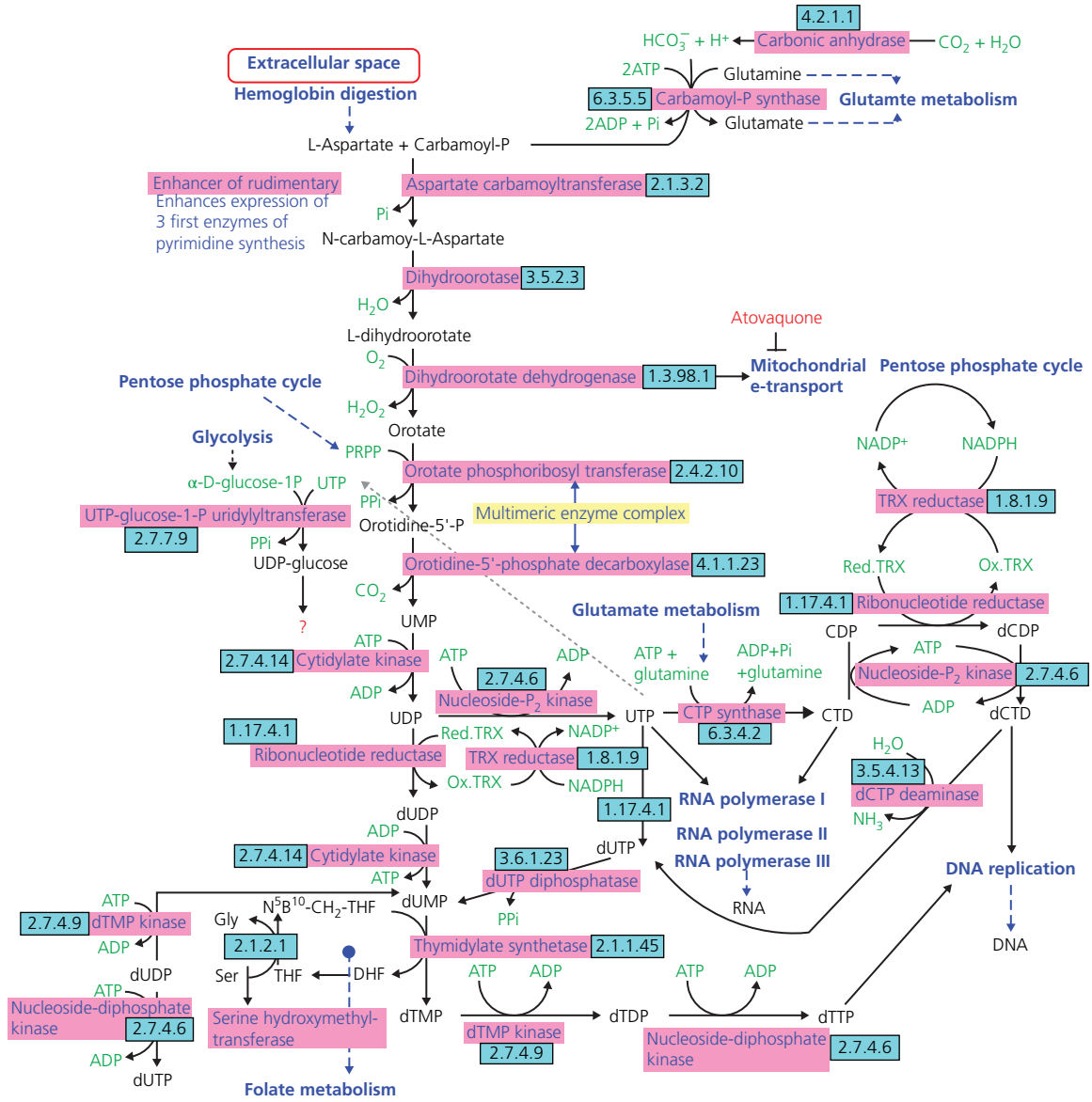


Figure 9.23 Pyrimidine biosynthesis. Pyrimidine nucleoside synthesis is allegedly *de novo*, starting from initial substrates provided by different metabolic pathways, while other substrates from yet other pathways are added along the way. The oxidation of L-dihydroorotate to orotic acid is coupled to the electron transport chain of the mitochondrion. As in purine metabolism, the reduction of nucleosides to deoxynucleosides is mediated by ribonucleotide reductase, which is then recycled by thioredoxin reductase.

unit to dUMP, which is catalyzed by thymidylate synthetase (EC 2.1.1.45). The production of CTP is the only known path for *de novo* synthesis of cytidine nucleotides (Reyes 1982). CTP synthase has been cloned, characterized, and functionally expressed (Hendriks 1998; Yuan 2005). The consideration of the pyrimidine pathway as a target for specific inhibitors has been reviewed (Hyde 2007; Cassera 2004).

Cofactors

CoA biosynthesis

Coenzyme A (CoA, CoASH, or HSCoA) is notable for its role in the synthesis of fatty acids and oxidation of pyruvate in the citric acid cycle. All genomes encode enzymes that use coenzyme A as a substrate, and around 4% of cellular enzymes depend on it or a thioester, such as acetyl-CoA, as a substrate. Coenzyme A is synthesized in a five-step process from pantothenate and cysteine (Figure 9.24), pantothenate being obtained from the extracellular medium (Saliba and Kirk 2001; Saliba 1998). Because coenzyme A is, in chemical terms, a thiol, it can react with carboxylic acids to form thioesters, thus functioning as an acyl group carrier, usually acetyl. Coenzyme A is also a source of phosphopantetheine group that is added as a prosthetic group to proteins such as acyl carrier proteins, essential and central for fatty acid biosynthesis in apicoplast and proteins functioning in similar manner such as formyltetrahydrofolate dehydrogenase. As discussed above, acetyl-CoA is produced during metabolization of phosphoenolpyruvate in apicoplasts, of pyruvate in mitochondria, and by the TCA cycle. Acetyl-CoA is also used for acetylation of histones and amino sugars. Human erythrocytes can synthesize CoA, and this synthesis is considerably increased in infected erythrocytes, where most of it is due to parasite's activity (Spry and Saliba 2009). A comprehensive survey on different inhibitors that act on acetyl CoA synthesis pathway has been published (Spry 2010).

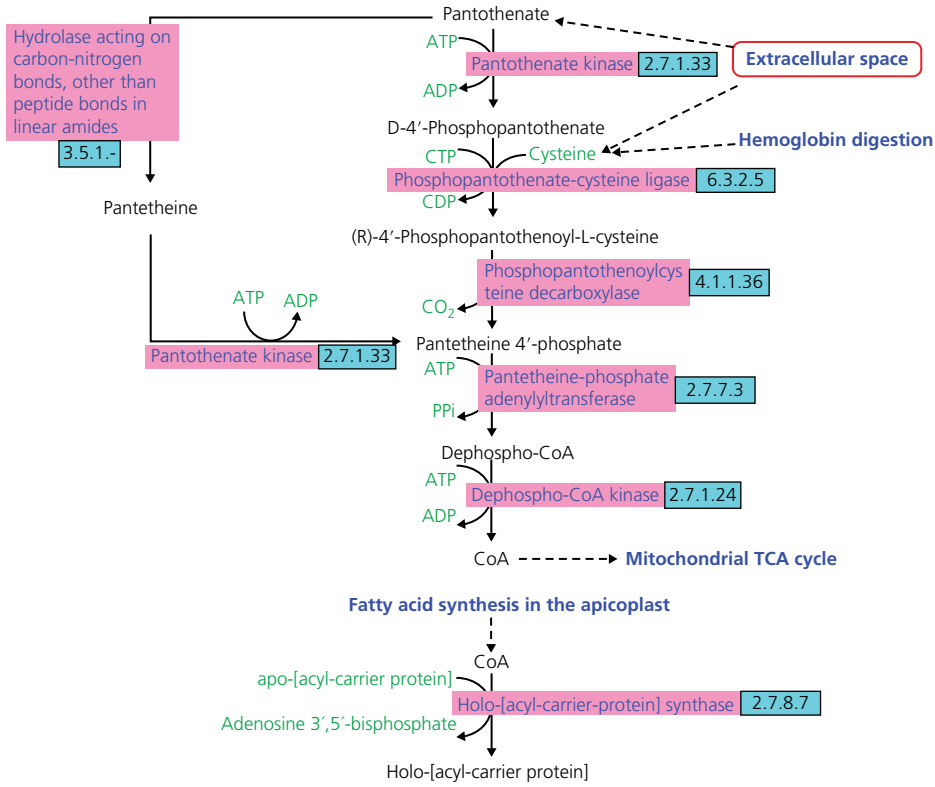


Figure 9.24 CoA biosynthesis. The synthesis of coenzyme A is totally dependent on the import of pantothenate from the extracellular space. This step, as well as some other enzymatic activities along the path, has been investigated to reveal some biochemical details.

Nicotinate and nicotinamide

Nicotinic acids are derivatives of pyridine that have a carboxy group, and nicotinamide is a nicotinic acid amide (niacin). Niacin is one of the five vitamins that when absent in the human diet are associated with a pandemic deficiency disease pellagra. As shown in Figure 9.25A, nicotinic acid is the substrate of NAD and NADP synthesis. NAD^+ and $NADP^+$ are coenzymes in a wide variety of enzymatic redox reactions and more specifically with 15 and 13 different parasite enzymes, respectively (Bozdech and Ginsburg 2005). Nicotinamidase is a hydrolytic enzyme involved in nicotinate and nicotinamide metabolism. The biochemical properties of nicotinamidase (EC 3.5.1.19) have been characterized to show a high affinity of 0.032 µM but relatively low k_{cat} of 0.30 s^{-1} (French 2010). However, mechanism of translocation of NAD(H) or NADP(H) in apicoplast or mitochondrion, where they are needed as cofactors, is not known.

Riboflavin biosynthesis

Riboflavin is the core component of cofactors FAD and FMN, and it is therefore required by all flavoproteins. Riboflavin deficiency inhibits growth of malaria parasites both *in vitro* and *in vivo*. The rate of uptake of [^{14}C] riboflavin and the subsequent biosynthesis of FMN and FAD were enhanced in infected erythrocytes compared to uninfected erythrocytes. Riboflavin uptake in erythrocytes

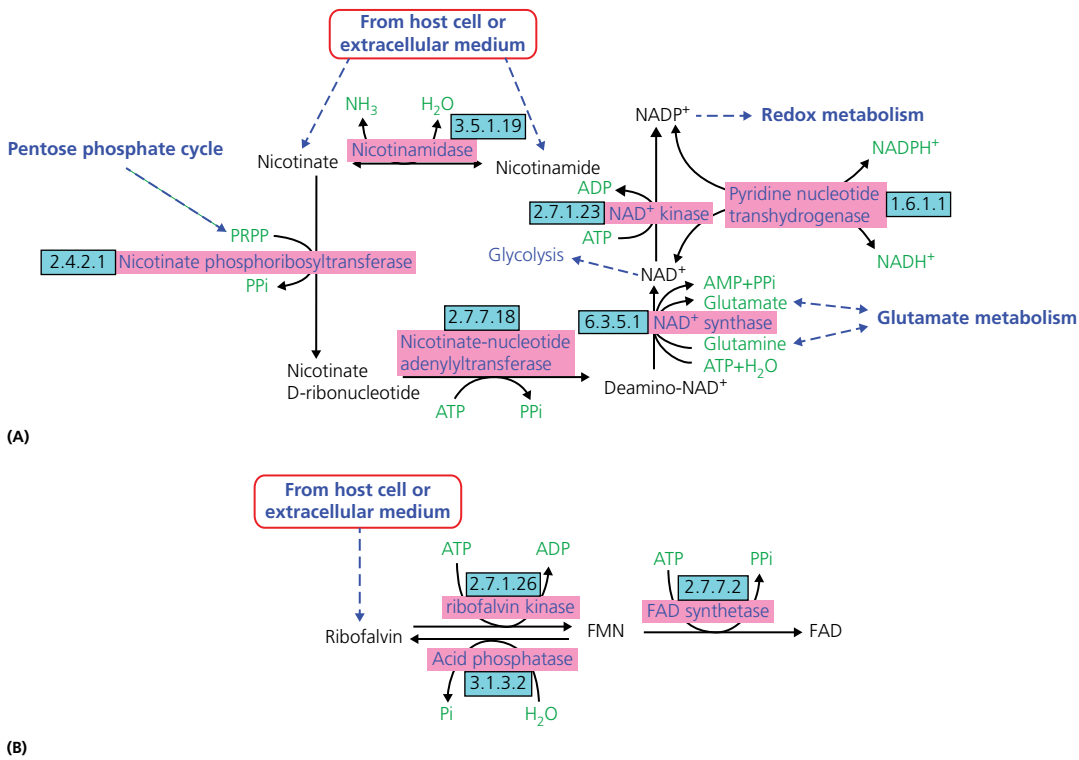


Figure 9.25 Nicotinate:nicotinamide and riboflavin metabolism. *A*, NAD and NADP, produced by the nicotinate and nicotinamide pathway, are essential co-factors for many redox reactions. Nicotinate and/or nicotinamide must be obtained from the extracellular space. Nicotinate is the first substrate of the pathway. *B*, Riboflavin is obtained from the host cell or from the extracellular medium. In one step it is converted to FMN, and a second enzymatic step produces FAD. Both FMN and FAD are co-factors in redox reactions.

was proportional to the level of parasitemia and was more intense in schizonts (Dutta 1991). As shown in Figure 9.25B, the production of flavin mononucleotide (FMN) or riboflavin-5′-phosphate from riboflavin is catalyzed by riboflavin kinase (EC 2.7.1.26). FMN functions as a prosthetic group of various oxidoreductases including NADH dehydrogenase (EC 1.6.5.3), as can be seen in the mitochondrial electron flow (Figure 9.33). During the catalytic cycle, the reversible interconversion of oxidized (FMN), semiquinone (FMNH•), and reduced ($FMNH_2$) forms occurs through the various oxidoreductases, which are able to play a part in both one- and two-electron transfers. FMN is a cofactor of chorismate synthase (Tapas 2011) and is the principal form of riboflavin found in cells and tissues. Its synthesis requires more energy, but it is more soluble than riboflavin.

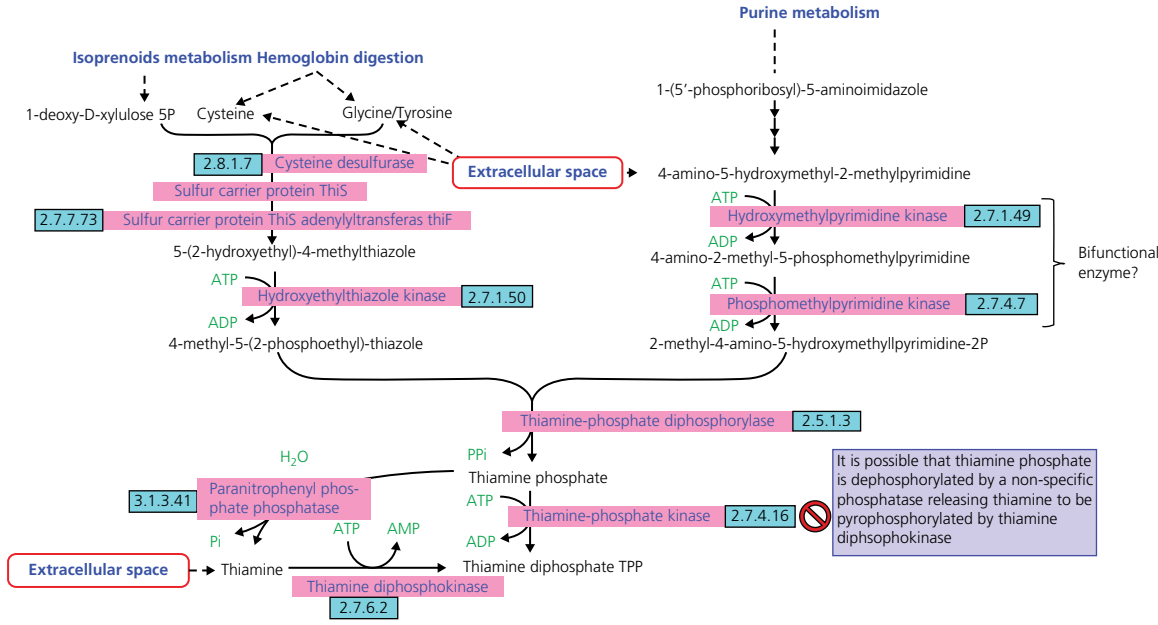
FAD is produced from FMN by FAD synthetase (EC 2.7.7.2;). It is a redox cofactor, which in its fully oxidized form (quinone) accepts two electrons and two protons to become $FADH_2$ (hydroquinone). $FADH_2$ can be oxidized to the semireduced form (semiquinone) FADH• by donating one electron and one proton. The semiquinone is then returned to original quinone form by losing an electron and a proton. $FADH_2$ in eukaryotes carries high-energy electrons used for oxidative phosphorylation. Any oxidoreductase enzyme that uses FAD as an electron carrier is called a flavoprotein. FAD is a prosthetic group in enzyme complex succinate dehydrogenase (EC 1.3.5.1; EC 1.3.99.1) that oxidizes succinate to fumarate in the citric acid cycle and in the mitochondrial glycerol-3-phosphate dehydrogenase (FAD^+) (EC 1.1.5.3) that oxidizes glycerol-3-phosphate to glycerone-phosphate (Mather and Vaidya 2008). The energy in $FADH_2$ is enough to produce 1.5 equivalents of ATP by oxidative phosphorylation. $FADH_2$ is also produced by β-oxidation, where FAD serves as a coenzyme to acyl CoA dehydrogenase. *P. falciparum* does not have either process. There are many flavoproteins (altogether, 24 are found in the genome of *P. falciparum*) besides the components of the succinate dehydrogenase complex, including α-ketoglutarate dehydrogenase, a component of the pyruvate dehydrogenase complex component E1 alpha subunit (EC 1.2.4.1) and of glutathione reductase (Krauth-Siegel 1996; Gilberger 2000).

Thiamine (Vitamin B_1)

Thiamine pyrophosphate (TPP) is the active form of vitamin B_1. It is a cofactor for various enzymes as shown in Figure 9.26. It is an essential cofactor of enzyme complexes such as pyruvate dehydrogenase, which resides in apicoplast, and for 2-oxoglutarate dehydrogenase, a major enzyme in the succinate dehydrogenase of the TCA cycle and in the conversion of pyruvate to acetyl-CoA by the BCKDH complex.

The biosynthesis of thiamine occurs by a combination of both pyrimidine and thiazole metabolic pathways. The pyrimidine branch (right branch in Figure 9.26) provides 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP), which is phosphorylated to HMP diphosphate by HMP/HMP-P kinase (EC 2.7.1.49). The thiazole branch produces 5-(2-hydroxyethyl)-4-methylthiazole phosphate. The biochemical properties of HMP/HMP-P kinase have been characterized (Wrenger 2006).

Subsequently, the respective phosphorylated pyrimidine and thiazole moieties, HMP-PP and THZ-P, are combined by thiamine-phosphate diphosphorylase (also named thiamine phosphate synthase; EC 2.5.1.3) to form thiamine phosphate (TMP). The additional phosphorylation by thiamine-phosphate kinase (EC 2.7.4.16) to form the active diphosphorylated form is unlikely because the gene encoding this enzyme has not been found in *P. falciparum* to date. It is possible that TMP is dephosphorylated by paranitrophenyl phosphate phosphatase (EC 3.1.3.41) (Knöckel 2008) before it is pyrophosphorylated by thiamine pyrophosphokinase (EC 2.7.6.2). Although TPP biosynthesis in the parasite has been demonstrated, it seems that *de novo* synthesis is not sufficient, and thiamine must be provided by the host cell inasmuch as removing thiamine and HMP from the culture medium is cytostatic to the parasite and their restitution restores parasite growth (Wrenger 2006).



TPP-dependent enzymes in *Plasmodium falciparum*

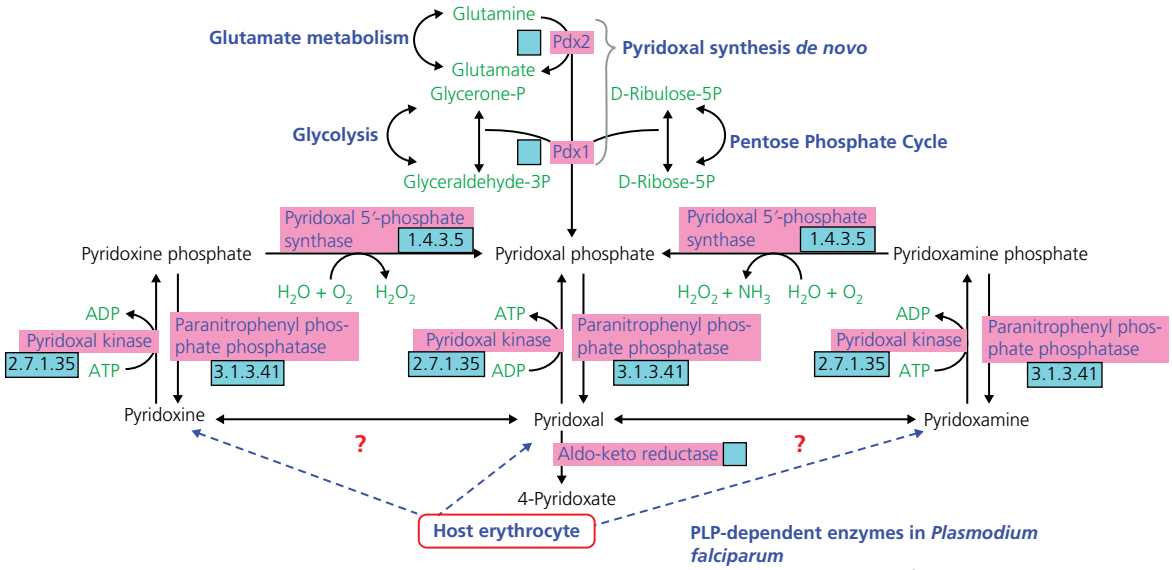
PfID	Enzyme name	
PF11_0256	Pyruvate dehydrogenase E1 alpha subunit	1.2.4.1
PF14_0441	Pyruvate dehydrogenase E1 beta subunit	1.2.4.1
PF08_0045	Oxoglutarate dehydrogenase	1.2.4.2
PFE0225W	3-methyl-2-oxobutanoate dehydrogenase	1.2.4.4
PF13_0070	2-oxoisovalerate dehydrogenase subunit α	1.2.4.4
MAL13P1.186	1-deoxy-D-xylulose phosphate synthase	2.2.1.7
PFF0530w	Transketolase	2.2.1.1

Figure 9.26 Thiamine (vitamin B_1) metabolism. As shown, thiamine pyrophosphate (TPP), which is the active form of vitamin B_1, is an indispensable co-factor for many essential enzymes (right, lower corner). Although thiamine can be obtained from the extracellular space an in one step, the parasite has the full complement of enzymes that are needed for the synthesis of TPP. The biosynthesis of thiamine occurs by the combination of the pyrimidine (right) branch and the thiazole (left) branch. In the absence of thiamine-phosphate kinase, thiamine-P is first dephosphorylated to thiamine and then rephosphorylated to TPP.

Thiamine cannot be synthesized by the human host, and although enzymes of the parasite biosynthetic pathways provide rational targets for drug development and several inhibitors have been investigated, none has emerged as a lead compound.

Pyridoxal phosphate (Vitamin B_6)

Vitamin B_6 consists of six molecules: pyridoxal, pyridoxine, pyridoxamine, and their phosphorylated forms. However, only pyridoxal 5-phosphate (PLP) is an active cofactor. PLP is known to serve about 150 enzymes as a cofactor. PLP serves as a carbonyl-reactive coenzyme in decarboxylation and transamination reactions involved in amino acid metabolism. *De novo* synthesis of PLP (Figure 9.27) uses intermediates from glycolysis and PPP and is catalyzed by a heteromeric glutamine amidotransferase consisting of a synthase subunit Pdx1 and a glutaminase subunit Pdx2. The full assembly contains 12 individual Pdx1/Pdx2 glutamine amidotransferases (Flicker 2007); the molecular details of the assembly have been characterized (Müller 2008). Pyridoxal kinase (EC 2.7.1.35) accepts other B_6 vitamers (pyridoxal, pyridoxine, and pyridoxamine), which can be



PLP-dependent enzymes in *Plasmodium falciparum*

Glycine hydroxymethyltransferase	2.1.2.1
5-Aminolevulinate synthase	2.3.1.37
Serine C-palmitoyltransferase	2.3.1.50
Aspartate aminotransferase	2.6.1.1
Ornithine aminotransferase	2.6.1.13
Branched-chain AA aminotransferase	2.6.1.42
Aromatic amino-acid transaminase	2.6.1.57
Cysteine desulfurase	2.8.1.7
Ornithine decarboxylase	4.1.1.17
Lysine decarboxylase	4.1.1.18
Phosphatidylserine decarboxylase	4.1.1.65
p-Aminobenzoic acid synthetase	4.1.3.38
Alanine racemase	5.1.1.1

Figure 9.27 Pyridoxal phosphate (Vitamin B_6). Pyridoxal 5-phosphate (PLP) is the active cofactor, known to serve ~150 enzymes (may be less in the parasite). Some examples in the parasite are shown in the lower right. The biosynthetic pathway uses intermediates from glycolysis, PPP, and glutamate. Pdx1/Pdx2 is a multimeric glutamine amidotransferase. Pyridoxal kinase accepts other B_6 vitamers (pyridoxal, pyridoxine, and pyridoxamine), which can be salvaged from the host erythrocyte.

salvaged from the host erythrocyte; its biochemical properties have been characterized (Wrenger 2005). Phosphorylation can be reversed by paranitrophenyl phosphate phosphatase (EC 3.1.3.41).

It has been reported that Pdx1/Pdx2-overexpressing cells have higher tolerance to singlet oxygen, thus confirming the existence of this oxidative radical and implying *de novo* vitamin B_6 synthesis as a component of the antioxidant defense machinery (Knöckel 2012). Some of the PLP-dependent enzymes have been studied in detail and are considered to be targets for the multipronged inhibition by specific enzyme inhibitors and that of PLP synthesis (Müller 2010).

Ubiquinone biosynthesis

Ubiquinones are electron carriers in mitochondrial electron transport. The quinoid nucleus of ubiquinone is derived from the shikimate pathway. 4-Hydroxybenzoate is directly formed from chorismate and all-*trans* geranyl-geranyl-PP (GGPP) through a series of modifications such as hydroxylations, decarboxylation, *O*-methylations, and methylations where the methyl groups are derived from *S*-adenosylmethionine. The side chain length of GGPP varies from 6 to 10 isoprene units. In eukaryotes, ubiquinones were found in the membranes of the mitochondria (inner

Figure 9.28 Ubiquinone biosynthesis. Ubiquinones are electron carriers in the mitochondrial electron transport. The quinoid nucleus of ubiquinone is composed of 4-hydroxybenzoate derived from chorismate and all-*trans*geranyl-geranyl-PP, and then undergoes a series of modifications such as hydroxylations, *O*-methylations, methylations, and decarboxylations. Note that genes encoding for two essential enzymes in the biosynthetic chain could not be found in the genome.

mitochondrial membrane), endoplasmic reticulum, Golgi vesicles, lysosomes, and peroxisomes, but their location in malaria parasites is unknown.

Ubiquinone biosynthesis was first reported in *Plasmodium lophurae* and in *P. knowlesi* (Rietz 1967; Skelton 1969). As shown in Figure 9.28, the biosynthesis of ubiquinone or coenzyme Q in *P. falciparum* involves two major steps: synthesis of the isoprene side chain by the terpenoid pathway and benzoquinone by the shikimate pathway. The synthesis of coenzymes Q8 and Q9 was studied by incorporation of [^{14}C]p-hydroxybenzoic acid (Schnell 1971). Coenzyme Q8 was most highly labeled, suggesting it to be the dominant species, which was further confirmed in a later study (Macedo 2010). When parasites were labeled with [1-^{14}C]IPP, [1-(n)-^{3}H]FPP, one major peak coincident with Q8 was obtained for all stages but was more evident for the schizont stage. With [1-(n)-^{3}H]GGPP, most of the label was incorporated in Q9. Thus, the parasite is able to synthesize different homologues of this molecule depending on the given intermediate.

As shown in Figure 9.28, no genes could be found for two enzymes of the ubiquinone biosynthesis pathway in the genome, namely, chorismate lyase (EC 4.1.3.40) and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (EC 4.1.1.-). However, since Q8 synthesis has been demonstrated, it is possible that relevant reactions are carried out by other enzymes or that the structure of the parasite enzymes has diversified so much during their evolution that they can no longer be detected by sequence similarity.

Porphyrin biosynthesis

Although the parasite is submerged in heme-containing Hb, it is unable to utilize it. Therefore, the parasite synthesizes it autonomously and probably produces all the heme it requires for viability (Surolia and Padmanaban 1992).

Heme is an iron-bound tetrapyrrole that serves as a prosthetic group in parasite cytochromes that participate in mitochondrial electron transfer (Surolia and Padmanaban 1991). The genes for all enzymes of heme-biosynthetic pathway (Figure 9.29) are located on the parasite genome, and subcellular location of their products has been demonstrated.

The first committed step of tetrapyrrole synthesis is the formation of δ-aminolevulinic acid (ALA), using glycine and succinyl-CoA in mitochondrion, catalyzed by aminolevulinate synthase (EC 2.3.1.37). The conversion of ALA to porphobilinogen is catalyzed by δ-aminolevulinate dehydratase (ALAD; EC 4.2.1.24). Import of host-derived ALAD was also shown to be essential for parasite heme synthesis and survival. Expression levels of PfALAD reveals that it may account for about 10% of the total ALAD activity in the parasite, the rest being accounted for by host enzyme imported by the parasite (Bonday 2000; Dhanasekaran 2004).The parasite ALAD gene encodes a bipartite presequence for apicoplast targeting (van Dooren 2002), and subcellular location has been confirmed (Dhanasekaran 2004).

Porphobilinogen deaminase(EC 2.5.1.61) and uroporphyrinogen III decarboxylase (EC 4.1.1.37) also have plastid-targeting presequences (Gardner 2002). The gene for uroporphyrinogen-III synthase (EC 4.2.1.75) could not be detected in the genome, but it has been demonstrated that this enzymatic function is carried out by porphobilinogen deaminase (Nagaraj 2008). Immunofluorescence studies suggested that coproporphyrinogen III oxidase (EC 1.3.3.3) is located in the cytosol (Nagaraj 2009, 2010a, 2010b).

On the basis of subcellular location and bioinformatic predictions, a hybrid model for heme biosynthesis has been proposed (Wilson 2005; van Dooren 2006). Thus, the hybrid pathway depicted in Figure 9.29 involves formation of porphobilinogen and coproporphyrinogen III in apicoplast, its translocation to cytoplasm be converted to protoporphyrinogen IX and then to mitochondrion again for conversion to heme. The model involves shuttling of pathway intermediates between mitochondria, apicoplast, and cytoplasm of parasite, with specific location of genome-encoded enzymes in different compartments. The rationale of evolution of this intricate pathway has been provided (Lim and McFadden 2010). The mechanism of translocation of heme intermediates between the two organelles remains to be clarified. Interestingly, supply of heme from digested hemoglobin to the newly synthesized heme has been demonstrated in *P. berghei* (Nagaraj 2013).

Shikimate metabolism

The shikimate pathway provides chorismate, the substrate for production of *p*-aminobenzoate, which is essential in the folate metabolic pathway. As shown in Figure 9.30, 3-deoxy-7-phosphoheptulonate synthase (EC 2.5.1.54), shikimate 5-dehydrogenase (EC 1.1.1.25), and shikimate kinase (EC 2.7.1.71) activities were identified in infected cells (Dieckmann and Jung 1986), but the genes encoding the first four enzymes could not be found in the *P. falciparum* genome.

The presence of the shikimate pathway in *Plasmodium* was made possible by the use of glyphosate (*N*-(phosphonomethyl) glycine), which is a highly effective herbicide due to its potent inhibition of 3-phosphoshikimate 1-carboxyvinyltransferase 5-enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19), which arrests parasite growth. This inhibition could be reversed by the addition of *p*- aminobenzoate and folate to the culture (Roberts 1998). Chorismate synthase does not have an inherent flavin reductase activity, and thus it is mono-functional, like the plant and bacterial

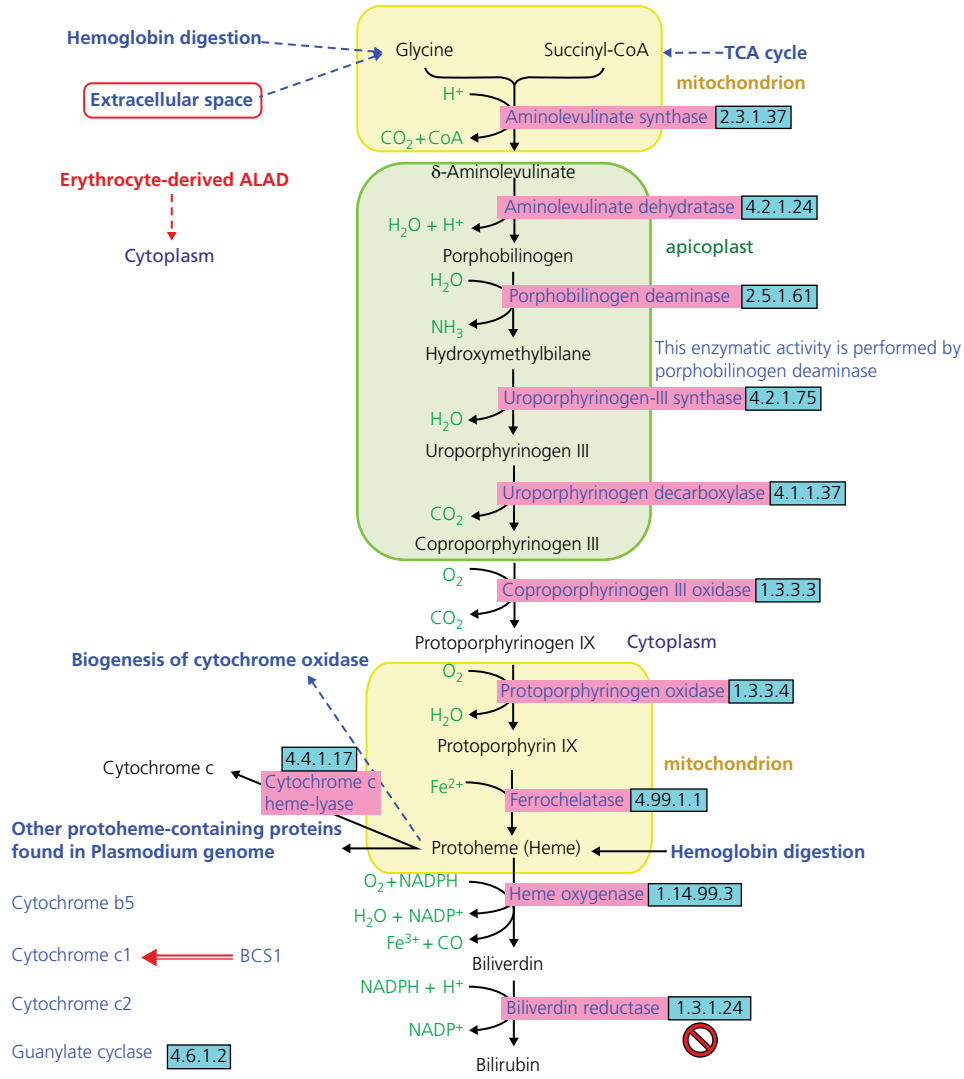


Figure 9.29 Porphyrin metabolism. The parasite cannot use the heme of the host cell Hb and synthesizes it, dividing the chore between the two juxtaposed organelles of the parasite, starting in the mitochondrion, then in the apicoplast; after a short sojourn in the cytosol, the path goes back to the mitochondrion for the final insertion of iron by ferrochelatase. Protoheme then serves as a prosthetic group in various cytochromes. Notice that aminolevulinatedehydratase (ALAD) has been shown to be imported from the host cell. Some heme is derived from hemoglobin digestion.

chorismate synthases (Fitzpatrick 2001). Shikimate kinase and 3-phosphoshikimate 1-carboxyvinyltransferase are amalgamated in a bifunctional enzyme.

The main challenge now is to resolve the enigma of missing genes that encode 3-deoxy-7-phosphoheptulonate synthase (EC 2.5.1.54), 3-dehydroquinate synthase (EC 4.2.3.4), 3-dehydroquinate dehydratase (EC 4.2.1.10), and shikimate 5-dehydrogenase (EC 1.1.1.25). Even an in-depth bioinformatics analytical study to identify the gene products has been unsuccessful (McConkey 2004).

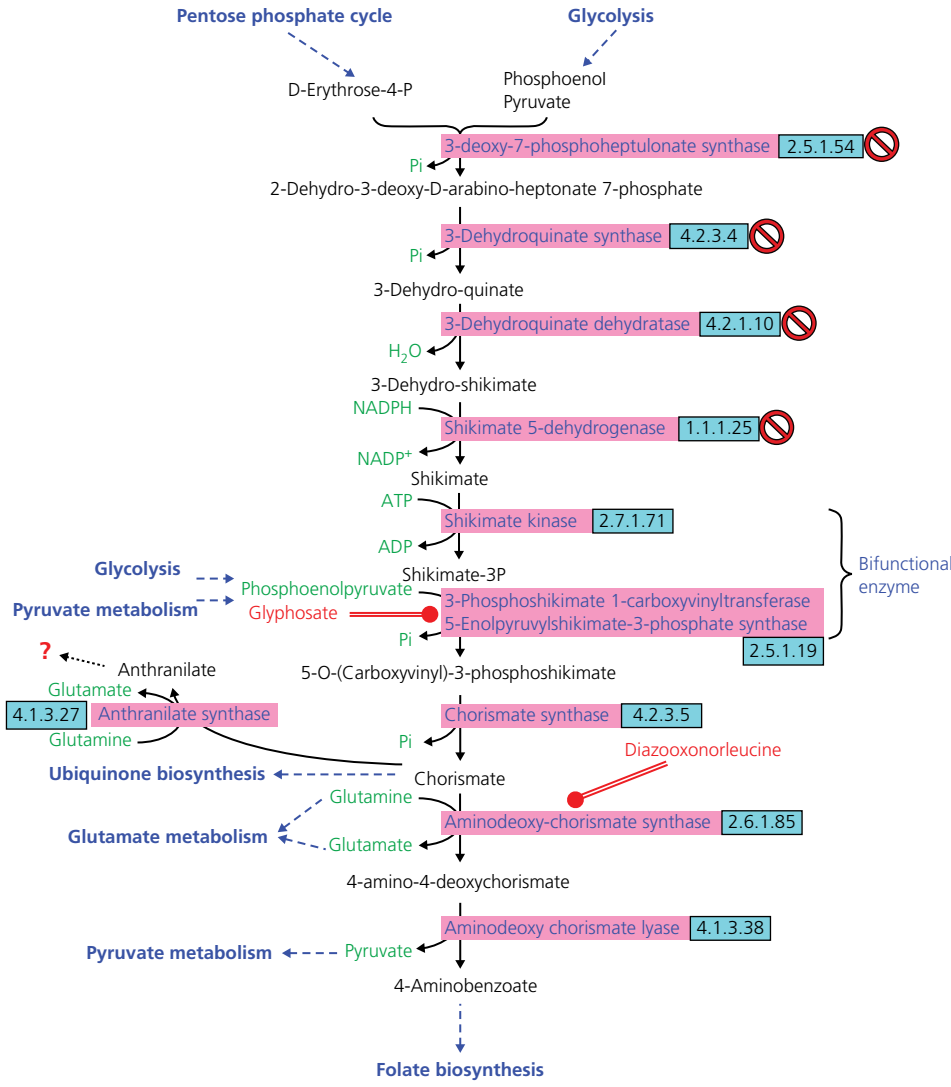


Figure 9.30 Shikimate biosynthesis. The products of the shikimate pathway serve for ubiquinone and folate synthesis, yet it poses one of the outstanding enigmas in the biochemistry of the parasite: for *four enzymes in a row,* no encoding genes could be found in the genome, and neither could an alternative pathway be identified. Yet the necessary intermediates that serve the depending pathways are there.

Folate metabolism

Tetrahydrofolate (THF) derivatives are essential cofactors for single carbon transfer reactions in the synthesis of nucleic acids and methionine. Unlike humans, who rely on dietary intake of preformed folates, *P. falciparum* is able to synthesize folate *de novo* by the condensation of pteridines, *p*-amino benzoic acid (PABA), and glutamate. Antifolate drugs targeting enzymes of folate biosynthesis have been effectively used in the treatment of *P. falciparum* malaria. Folate metabolism provides reduced folate cofactors that serve in a variety of reactions involving the transfer of one-carbon units. Folate cofactors are needed in one-carbon unit transfers such as the synthesis of deoxythymidylate (dTMP) that is required for DNA synthesis, formation of L-methionyl-tRNA (fMet), and other processes.

As shown in Figure 9.31, biosynthesis of folate moieties starts with GTP and is catalyzed by five enzyme activities: GTP cyclohydrolase (EC 3.5.4.16); 6-pyruvoyltetrahydropterin synthase (EC 4.2.3.12), which is an atypical orthologue of 6-pyruvoyltetrahydropterin synthase and has been shown to offer the missing link in the pathway (Dittrich 2008); hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3), which together with dihydropteroate synthase (EC 2.5.1.15) forms a bi-functional enzyme that incorporates *p*-aminobenzoate ; finally, dihydrofolate synthase (EC 6.3.2.12) forms dihydrofolate (H_2folate), which is reduced to a biochemically active form, tetrahydrofolate, by dihydrofolate reductase (EC 1.5.1.3). Following the actions of serine hydroxymethyltransferase (SHMT,EC 2.1.2.1), thymidylate synthase (EC 2.1.1.45), the resulting H_2folate reenters the cycle in order to ensure continuous synthesis of dTMP.

H_4folate exists mainly in polyglutamated forms (Krungkrai 1989), which are preferred substrates for folate-utilizing enzymes (Krumdieck 1992). Folylpolyglutamate synthase (EC 6.3.2.17) is a bifunctional enzyme along with dihydropteroate synthase (Wang 2010). It is not known whether folate is polyglutamated at either the H_2folate or H_4folate stage. Serine hydroxymethyltransferase requires the cofactor pyridoxal 5′-phosphate for enzymatic activity and to bind with its cognate mRNA. RNA binding could not be reversed by enzyme substrates. However, the RNA binding of SHMT was not tight enough to inhibit protein translation (Pang 2009). The catalytic properties of the enzyme have been determined. The K_ms for serine and H_4folate were considerably higher and the k_{cat} was much lower compared to human and *E. coli* enzymes.

Some cultured parasites are also capable to salvage preformed folates from the host (Wang 1999). *p*-Aminobenzoic acid, the substrate of dihydropteroate synthase, is obtained from the host via a salvage mechanism (Wang 2004). Although parasites may also derive PABA from chorismate catalysed by aminodeoxychorismate synthase (EC 2.6.1.85) and aminodeoxychorismate lyase (EC 4.1.3.38). However, no encoding gene could be detected in the genome for these two enzymes. Both folic acid derivatives and *p*-aminobenzoate are imported from the host cell through two parasite cell membranes by dedicated folate transporters (Salcedo-Sora 2011).

Due to space restrictions, the action of drugs on specific targets of the folate pathway, molecular biology and biochemical studies are not reviewed in this chapter. Thorough reviews of these topics are available (Hyde 2005; Yuthavong 2006). Further research is required to elucidate the drug resistance mechanisms through folate pathway targets because the sulfadoxine–pyrimethamine combination is successfully used for intermittent prophylactic treatment within the context of malaria elimination.

Redox metabolism

The intraerythrocytic parasite produces superoxide during the digestion of the host cell oxyhemoglobin (oxyHb) in the food vacuole (Atamna and Ginsburg 1993). This probably explains the microaerophilicity of the parasites in culture and its adherence to the postcapillary venules, because the ambient oxygen level determines the extent of oxyHb and parasite response (Briolant 2007). The superoxide anion dismutates into H_2O_2 in the acidic pH of the vacuole. A part of it may be detoxified by ingested host cell catalase, but most of it exits into the parasite, where it is partially handled by the parasite's antioxidant defense system. However, part of it reaches the host cell, where it inflicts various damages (Turrini 2003). There are other sources for production of reactive oxidative species, such as the electron-transport chain of the mitochondrion.

As shown in Figure 9.32, the parasite is equipped with different antioxidant molecules and enzymes to cope with the oxidative stress. The parasite synthesizes glutathione (GSH) and at some stage even supplies it to the incapacitated host cell (Atamna and Ginsburg 1997). Inhibition of GSH

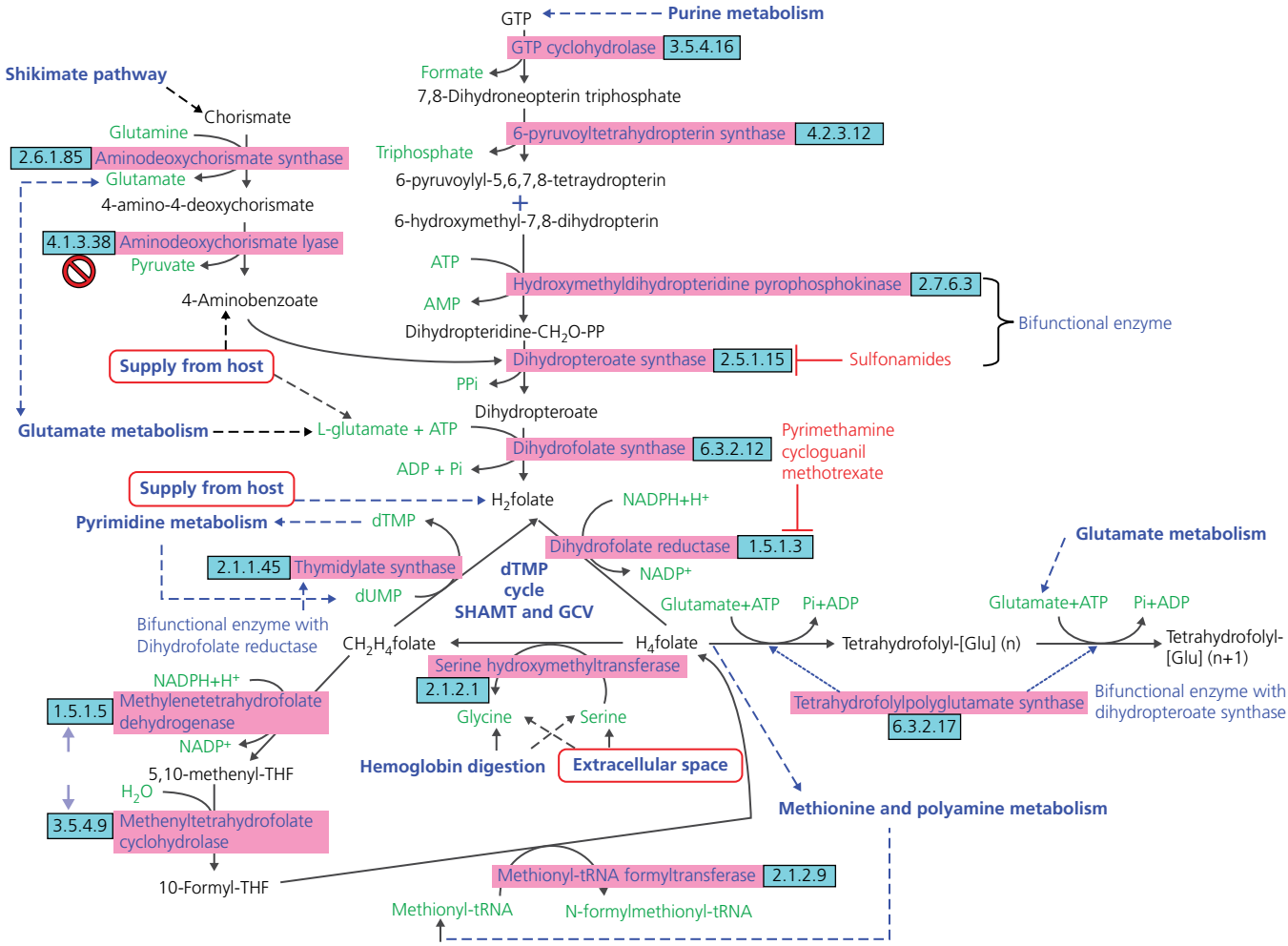


Figure 9.31 Folate biosynthesis. The folate pathway generates tetrahydrofolate (THF) essential cofactors for single carbon-transfer reactions in the synthesis of nucleic acids. The pathway, which is absent in humans, has been the target of antimalarial chemotherapy. Some drugs are shown next to their targets.

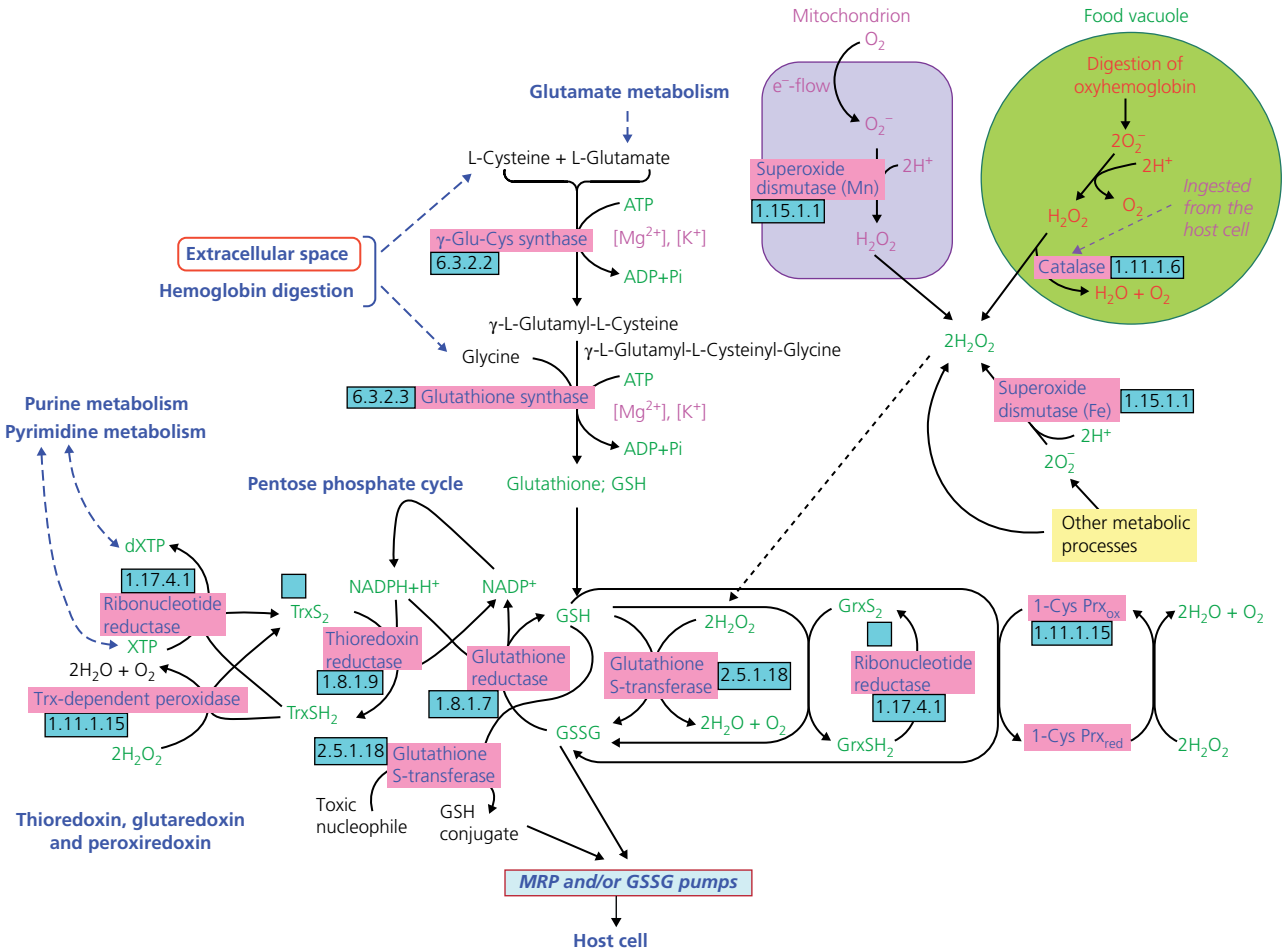


Figure 9.32 Redox metabolism. An intricate network of antioxidant defense is put in place in order to defend the parasite from intrinsic and extrinsic oxidative challenges. The system consists of glutathione, thioredoxin, and peroxiredoxin and their redox cycling, mostly through NADPH, which is recycled mostly by the PPP.

synthesis by D,L-buthionine (S,R)sulfoximine (BSO), an inhibitor of gamma-glutamyl-cysteine synthetase (EC 6.3.2.2), inhibits parasite growth (Meierjohann 2002). The fact that the parasite does not have a glutathione peroxidase (Sztajer 2001) but depends on GSH and its synthesizing enzymes essential for parasite survival (Patzewitz 2012), indicates that this tripeptide has other roles.

GSH takes part in detoxifying heme, a product of hemoglobin digestion (Famin 1999; Ginsburg 1998); it provides electrons for deoxyribonucleotide synthesis by ribonucleotide reductase (EC 1.17.4.1); it is a substrate for glutathione *S*-transferase (EC 2.5.1.18), whereby GSH is conjugated to nondegradable compounds, including antimalarial drugs; and it is a cofactor in glyoxalase metabolism that detoxifies methylglyoxal. GSSG produced during GSH-assisted reduction is recycled by means of glutathione reductase (EC 1.8.1.7) using $NADPH^+$ recycled by the pentose phosphate pathway. The biochemical parameters of glutathione reductase were found to be different in chloroquine-sensitive versus chloroquine-resistant strains, and the molecular attributes of these changes were identified (Gilberger 2000). Some GSSG may be exported outside of the parasite by the MRP (multidrug resistance–associated proteins) and/or GSSG pumps. GSH has been shown to derivatize several hundred parasite proteins (Kehr 2011), some of which belong to the antioxidant defense battery. Some enzymes were shown to be reversibly glutathionylated, suggesting a regulatory role for the modification. Glutaredoxin 1, thioredoxin 1, and plasmoredoxin are able to efficiently catalyze protein deglutathionylation.

The thioredoxin (Trx) system of the parasite is the major antioxidant system. Thioredoxins are small redox-active proteins containing an active-site dithiol motif. Present at micromolar concentrations, thioredoxins play a major role in reducing protein disulfides via their specific dithiol/disulfide active site. The parasite is endowed by a functional Trx system, composed of NADPH, Trx reductase (EC 1.8.1.9), several thioredoxins and Trx-like proteins, Trx-dependent peroxidases (glutaredoxins; EC 1.11.1.15), and plasmoredoxin. Several of these components were characterized for their biochemical and molecular features, including their crystal structures (Deponte 2005; Banerjee 2009).

An investigation of the subcellular location of antioxidant defense-related proteins (Kehr 2010) revealed the importance of antioxidant components in the mitochondrion, apicoplast, and endoplasmic reticulum and the use of alternative translation initiation to achieve differential subcellular targeting.

A plant-type redox system consisting of ferredoxin-$NADP^+$ reductase (EC 1.18.1.2) and its redox partner, ferredoxin, have been found in the apicoplast (Kimata-Ariga 2007a). The 3D-structure of the enzyme has been determined and the molecular details of their interactions with ferredoxin have been investigated (Kimata-Ariga 2007b). The redox properties of the enzyme were determined and showed that the FAD semiquinone species is highly destabilized. It was proposed that the slow hydride transfer and unstable FAD semiquinone are responsible for the poor catalytic efficiency of the enzyme (Balconi 2009). Genes encoding mitochondrially targeted orthologues are found in the genome. This redox system plays a crucial role in the biosynthesis of iron-sulfur–containing proteins in both organelles (Seeber 2005).

Mitochondrial functions

Mitochondrial electron flow

In most eukaryotic cells, the mitochondrial electron transport chain (ETC) is used for the generation of a proton motive force, which is essential for oxidative phosphorylation to produce ATP. However, in the erythrocytic stage of malaria parasites, as in other organisms or cell types that favor glycolysis,

the role of the ETC may be considerably reduced (van Raam 2008), and mitochondria may even develop into consumers rather than producers of ATP (Traba 2008).

The ETC of *P. falciparum* has been reviewed (Vaidya and Mather 2009) and is shown in Figure 9.33. Evidence for a functional classical respiratory pathway includes the existence of a mitochondrial transmembrane potential (Divo 1985) and substrate-dependent mitochondrial oxygen consumption (Fry and Beesley 1991). The parasite has five enzymatic activities for the respiratory chain: NADH dehydrogenase (complex I; EC 1.6.5.3), succinate dehydrogenase (complex II; EC 1.3.5.1 and 1.3.99.1), ubiquinol–cytochrome *c* oxidoreductase (complex III; EC 1.10.2.2), cytochrome *c* oxidase (complex IV; EC 1.9.3.1), and ATP synthase (complex V; EC 3.6.3.14) with ubiquinone (UbQ) and cytochrome *c* functioning as electron shuttle between the complexes. The proton motive force is generated by complexes I, III, and IV.

Parasites have lost the large multisubunit complex I, although the activity of NADH dehydrogenase, a non–proton-pumping single subunit that reduces ubiquinone, could be measured (Krungkrai 2002). It has been reported that the parasite holds a type II NADH dehydrogenase instead of the conventional rotenone-sensitive complex I (Biagini 2006). During aerobic respiration, the flavoprotein and the iron sulfur protein subunits of complex II join together (Takeo 2000) to catalyze the electron transfer from succinate to quinone. In the absence of oxygen, the electrons transfer from quinol to fumarate (Suraveratum 2000).

The product of the gene encoding complex III was characterized (Learngaramkul 1999) and found to be sensitive to atovaquone, myxothiazole, and antimycin, which collapse mitochondrial membrane potential and inhibit respiration. They did not induce apoptosis but rather a noticeable static state (Painter 2010).

The ETC of the parasite's mitochondrion is known to establish a membrane potential, but it is not known if a proton gradient is also formed. Both are essential driving forces for the F_0F_1-ATP synthase (complex V; EC 3.6.3.140). Most of the subunits of this multimeric complex have been identified either *in silico* or experimentally (Mogi and Kita 2009). It has been demonstrated that the molecular mass of the ATP synthase complex exceeds 1000 kDa (one million Daltons), consistent with a dimer, which indicates the presence of hitherto unidentified subunits in addition to the canonical subunits in the parasite ATP synthase complex. Attempts to disrupt the genes encoding β and γ subunits failed, implying that the ATP synthase plays an essential role in the erythrocytic stages (Balabaskaran 2011).

Summarizing ETC functions, it can be stated that the critical function of the mitochondrial ETC is the regeneration of UbQ as a cosubstrate of DHODH. The survival of the parasite in the presence of ETC inhibitors, when DHODH is superfluous, indicates that the other UbQ-requiring mitochondrial dehydrogenases such as succinate dehydrogenase, NADH dehydrogenase, glycerol-3-phosphate dehydrogenase (FAD^+; EC 1.1.5.3), and malate-quinone oxidoreductase (EC 1.1.99.16) are not essential for parasite growth during the erythrocytic phase. It has been demonstrated that the requirement for the ETC may be strain specific and that ubiquinone-dependent dehydrogenase activities other than those of DHODH are dispensable in some strains but are essential in others (Ke 2011).

The potential of the components of the ETC to serve as drug targets has been reviewed (Torrentino-Madamet 2010).

Mitochondrial TCA cycle

The acristate structure and low oxygen consumption of the parasite's mitochondrion casts doubt over its ability to generate ATP by oxidative phosphorylation. Although the genes encoding all TCA cycle enzymes (Gardner 2002) and ATP synthase subunits are present in the parasite genome

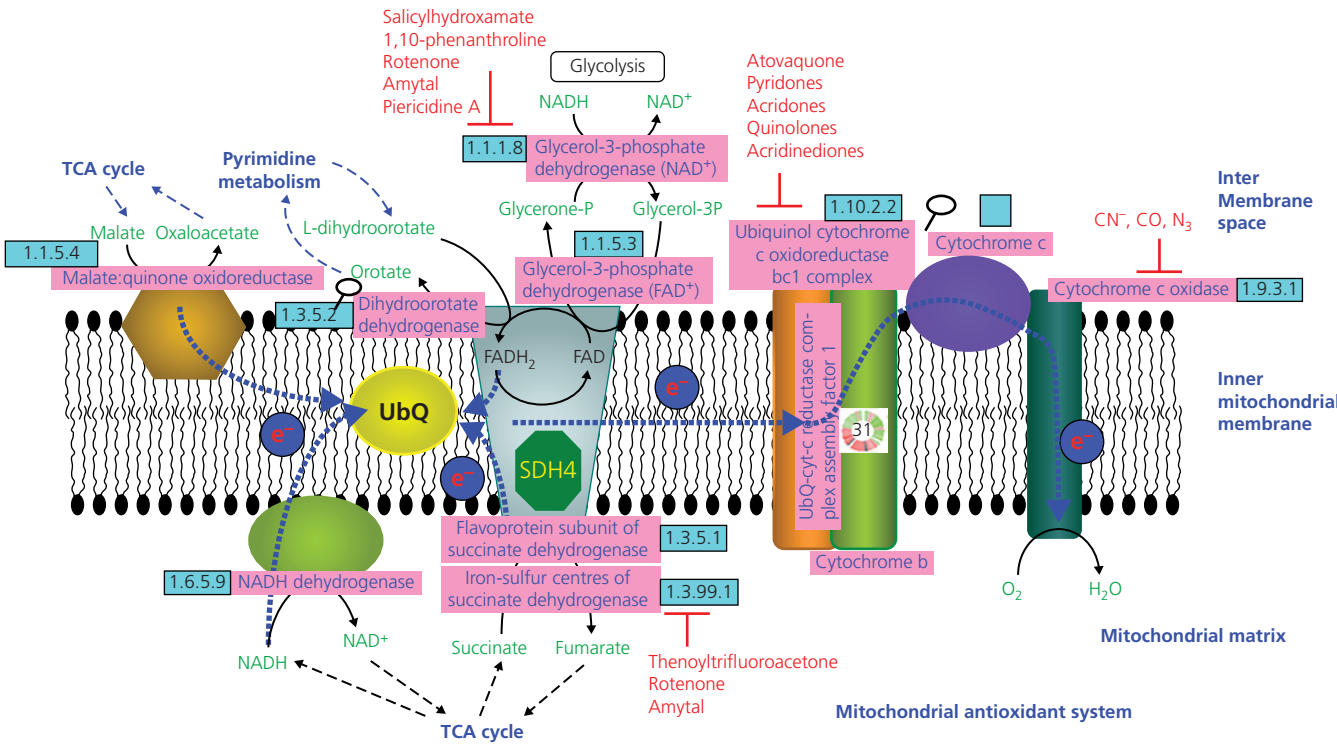


Figure 9.33 Mitochondrial electron flow (ETC). Because oxidative phosphorylation could not be demonstrated in the parasite, the role of the ETC is considerably reduced, and its main function is the removal of electrons from dihydroorotic acid during its oxidation to orotate. However, new functions seem to emerge explaining the roles of the various dehydrogenases that are linked to the TEC (see text).

(see the above section on the ETC), oxidative phosphorylation has never been detected. The mitochondrion is also exempt from other classical roles of its counterparts in other cells, such as involvement in amino acid synthesis and β-oxidation of fatty acids. It seems that the role of the *P. falciparum* TCA cycle is to produce succinyl-CoA for *de novo* heme biosynthesis, NADH for the ETC, and acetyl-CoA.

The TCA enzymes citrate synthase (EC 2.3.3.1), aconitase (EC 4.2.1.3), and dihydrolipoyl dehydrogenase (EC 1.8.1.4) have been located in the mitochondrion (Tonkin 2004; Hodges 2005; McMillan 2005). Biochemical characterization of the TCA cycle enzymes succinate dehydrogenase (EC 1.3.99.1) and aconitase was performed on parasite extracts, and IDH has been cloned and characterized (Chan and Sim 2003; Wrenger and Müller 2003). The production of succinyl-CoA alludes to the function of two other cycle enzymes, 2-oxoglutarate dehydrogenase (EC 1.2.4.2) and succinyl-CoA synthetase (EC 6.2.1.4 and 6.2.1.5). EC 6.2.1.4 produces GTP and CoA, and EC 6.2.1.5 produces ATP and CoA. 2-Oxoglutarate dehydrogenase is a multienzyme complex containing α-ketoglutarate dehydrogenase, dihydrolipoamide *S*-succinyl transferase (EC 2.3.1.61), and dihydrolipoyl dehydrogenase (EC 1.8.1.4). A metabolomic survey revealed oscillating levels of several TCA cycle intermediates, 2-oxoglutarate, citrate, isocitrate, and *cis*-aconitate, suggesting that they are actively synthesized (Olszewski 2009) Figure 9.34.

The mitochondrion is devoid of pyruvate dehydrogenase (Foth 2005), an enzyme essential for the conversion of pyruvate to acetyl-CoA to produce citrate and thus fuel the TCA cycle. However, as previously suggested, acetyl-CoA could be produced in the mitochondrion by the mitochondrion-located branched-chain α-keto acid dehydrogenase (BCKDH) complex, which uses pyruvate produced in glycolysis (Seeber 2008). This has now been shown experimentally (Cobbold 2013). Thus, glycolysis-derived pyruvate enters into the mitochondrion and is metabolized to acetyl-CoA. Interestingly, an essential TCA enzyme, malate dehydrogenase, was located in the cytosol (Lang-Unnasch 1992), but the mitochondrial function of the enzyme could also be fulfilled by malate:quinone oxidoreductase. Nevertheless, a functional mitochondrial MDH could be inferred from metabolomic studies.

Advances in mass spectrometry-based high-throughput chemimetric technologies allow addressing this enigma. Rapid and simultaneous quantification of many cellular metabolites allow metabolic tracing experiments using stable isotope-labeled substrates in which the number and position of labeled atoms are determined (MacRae 2013; Cobbold 2013). Both research groups fed parasite cultures with ^{13}C-glucose and ^{13}C-glutamine, and Cobbold's group also used [2-^{13}C]acetate and [1-^{13}C]pyruvate. The analysis of isotopic distributions in the infected cells revealed that carbon skeletons derived from both glucose and glutamine are catabolized in a canonical oxidative TCA cycle in both the asexual and sexual blood stages. All glycolytic intermediates were uniformly labeled, as well as some TCA cycle intermediates. "The predominant isotopomers of citrate in ^{13}C-glucose fed infected RBC contained +2, +4 or +6 labeled carbons, indicating the operation of a canonical TCA cycle in which pyruvate feeds into the cycle via acetyl-CoA. Citrate isotopomers containing +3 and +5 labeled carbons were also detected, reflecting the activity or activities of the plasmodium PEP carboxylase (PEPC) and/or PEP carboxykinase (PEPCK) that catalyze the carboxylation of $^{13}C_3$-phosphoenolpyruvate (PEP) to $^{13}C_3$-oxaloacetate" (McRae 2013) (see Pyruvate metabolism, above).

Labeling of all TCA cycle intermediates was also observed using ^{13}C-glutamine to feed the *P. falciparum* asexual stages. The predominant isotopomers of succinate, malate, and fumarate were fully labeled, indicating that most of the carbon skeletons that enter the TCA cycle via glutamate are not continuously cycled through the TCA reactions but are presumably exported from the mitochondrion. Jointly, these investigations show that the parasite uses both pyruvate and glutamate in an

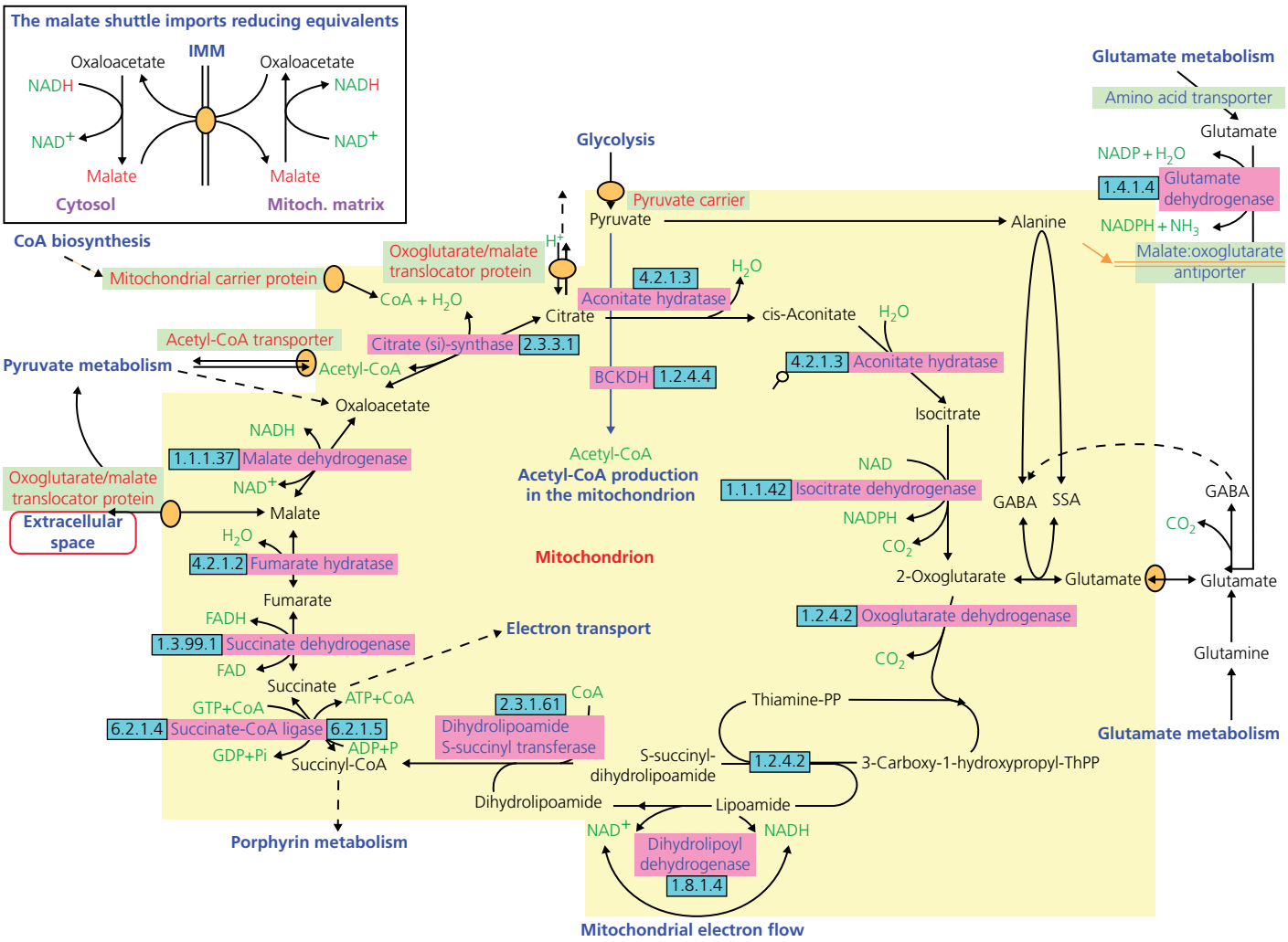


Figure 9.34 Mitochondrial TCA cycle. All genes encoding TCA enzymes and the ancillary transporters are found in the genome. Metabolomic studies using stable isotope-labeled substrates in which the number and position of labeled atoms are determined have revealed that essentially a typical TCA cycle is operating, although it uses glutamate rather than pyruvate as its main substrate. Results also showed that the BCKDH complex is able to convert pyruvate to acetyl-CoA (see Figure 9.35). The major products are acetyl-CoA used for various acetylation reactions, succinyl-CoA used in the synthesis of porphyrin and NADH, which fuels the mitochondrial electron transport. The malate shuttle (inset) is able to transfer reducing equivalents from the cytosol to the mitochondrial matrix.

unoriginal TCA. They also indicate some degree of partitioning within the cycle, with glutamate sustaining a major flux from 2-oxoglutarate to malate/oxaloacetate, and with glucose-originated pyruvate and oxaloacetate contributing to a minor flux toward citrate synthesis.

Remarkably, gametocyte-infected RBCs used ^{13}C-glucose at a rate exceedingly greater than did uninfected RBC. ^{13}C-acetate was a major product of gametocytes, indicating higher conversion either by apicoplast or by mitochondrion.

The flux of glucose carbon skeletons into the TCA cycle is low in the asexual blood stages but increases dramatically in the gametocyte stages. Glutamine is the major provider of carbon skeletons in the asexual stages. The apicoplast PDH is neither a substantial contributor nor essential to acetyl-CoA metabolism. Most of the acetyl-CoA is provided by the mitochondrial BCKDH and by cytosolic ACS (see Pyruvate metabolism, above) in blood-stage parasites. Conspicuously, the parasite displays significant stage-dependent metabolic changes during their intraerythrocytic developmental cycle.

The metabolomic data suggested two independent pathways for the production of acetyl-CoA: the mitochondrial glutamine-derived acetyl-CoA providing for histone acetylation and the cytosolic glucose-derived acetyl-CoA, which is used to acetylate amino sugars. This logistic is rather problematic because it is hard to explain how acetyl-CoA is compartmentalized after it has been produced and released into the cytosol.

Toxoplasma gondii encodes a putative glutamate/GABA transaminase and other proteins needed for synthesis of GABA and import of this metabolite into the mitochondrion (McRae 2012). If this were also the case in *P. falciparum*, it could explain the observation that GABA is synthesized in both asexual and sexual RBC stages and that pyruvate is converted to alanine in agreement with the notion that both metabolites could contribute to transamination reactions in the mitochondrion, which could convert glutamate to 2-oxoglutarate (McRae 2013).

Acetyl-CoA production in the mitochondrion

The bulk of the acetyl-CoA pool is not changed in PDH-deficient parasites, as attested by TCA intermediates, fatty acid profiles, or viability of blood-stage parasites (Cobbold 2013). PDH deficiency is expressed only at the mosquito stage. The production of acetyl-CoA is fully accounted for by the mitochondrial branched chain α-keto (BCKDH) complex and by cytoplasmic acetyl-CoA. While BCKDH supplies acetyl-CoA to the TCA cycle, ACS donates to the acetylation of amino.

All subunits of BCKDH, E1α, E1β, E2 and E3 are encoded by the *P. falciparum* genome. The mitochondrion is devoid of pyruvate dehydrogenase (Foth 2005), an enzyme essential for the conversion of pyruvate to acetyl-CoA to produce citrate and thus fuel the TCA cycle. The function of BCKDH was previously suggested to produce acetyl-CoA in the mitochondrion by the mitochondrion-located branched-chain α-keto acid dehydrogenase, using pyruvate produced in glycolysis (Seeber 2008). This has now been shown experimentally (Cobbold SA 2013). Thus, glycolysis-derived pyruvate enters into the mitochondrion and is metabolized to acetyl-CoA (Figure 9.35).

Hemoglobin digestion and hemozoin production

Hemoglobin (Hb) degradation in intraerythrocytic malaria parasites is an intense process that occurs in the acidic environment of the digestive or food vacuole (FV). As shown in Figure 9.36, the process starts with the endocytosis of host cell cytosol, which involves both the parasitophorous and

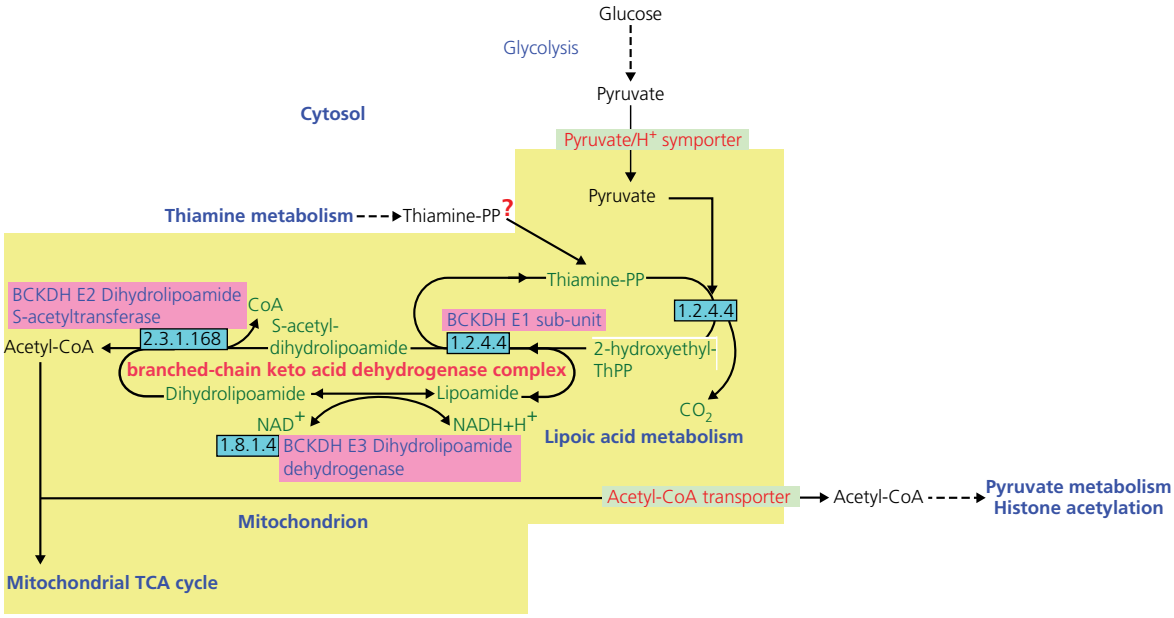


Figure 9.35 Acetyl-CoA production in the mitochondrion. The mitochondrion-located branched-chain α-keto acid dehydrogenase complex (BCKDH) converts pyruvate to acetyl-CoA. It uses the same E3 dihydrolipoamide dehydrogenase as the TCA cycle but has particular E1 and E2 subunits.

the plasma membranes (Yayon 1984). Polyphosphorylated phosphoinositides (PIPs) of parasite origin are involved in this process inasmuch as phosphoinositides and their specific kinases are seen in various subcellular sites involved in protein trafficking. PfPI3K is involved in endocytosis from the host and trafficking of Hb in the parasite, and PIP3 was found associated mainly with the membrane of the food vacuole and the apicoplast (Vaid 2010; Tawk 2010).

The endocytic vesicles fuse with the membrane of the FV and release their cargo therein. Two types of proton pumps, one ATP- and one pyrophosphate-fueled (Saliba 2003) are located in the FV membrane to acidify the lumen of the FV to a pH of about 5.5 (Klonis 2007). In the lumen of the FV, endopeptidases of diverse catalytic mechanisms and specificity are present. These include aspartic proteases, plasmepsins I, II, and IV, histo-aspartic protease, and the cysteine proteinases falcipain-2, -2′, and -3, and falcilysin. Their major substrates are the α- and β-globin chains, which are digested to oligopeptides that are further hydrolyzed to dipeptides and amino acids. Both egress through yet -unidentified transporters of the FV membrane. Hb digestion is most active at the late ring and trophozoite stages (Gavigan 2001).

The proplasmepsins are synthesized as integral membrane proteins that are transported to the food vacuole in an indirect way: They are first secreted through the plasma membrane of the parasite to the host cell, where they are cleaved to a soluble form (Francis 1997; Klemba 2004). Eventually, they are taken up into the vesicles that form at cytostomes and that carry host cell cytosol to the food vacuole. Proplasmepsin maturation appears to require acidic conditions (Francis 1997) and occurs principally via the falcipains (Drew 2008). But if falcipain activity is inhibited, autoprocessing can take place helping as an alternate activation system. These results establish some redundancy between the protease families involved in *Plasmodium* Hb degradation.

Hemoglobin digestion

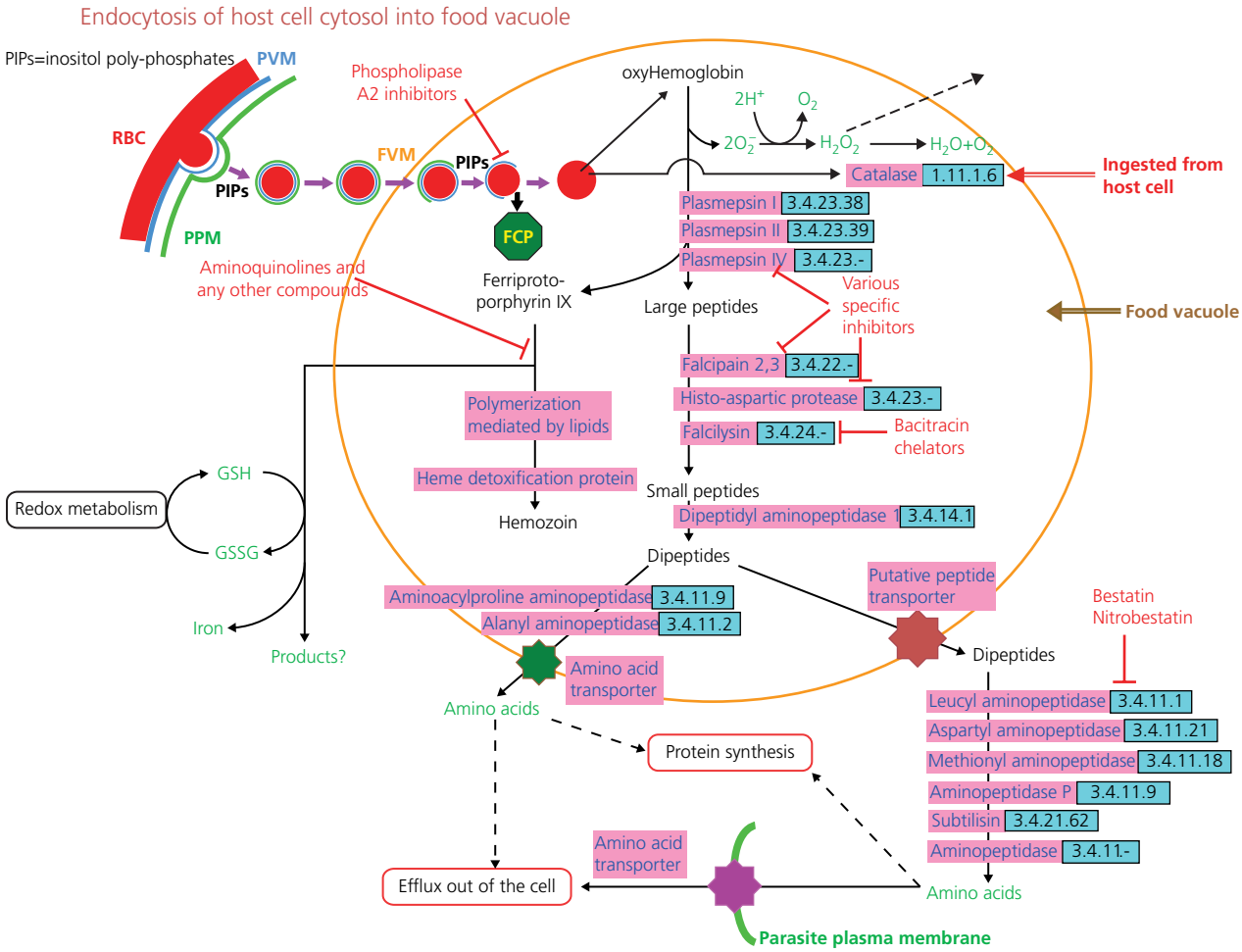


Figure 9.36 Hemoglobin digestion and ferriprotoporphyrin IX polymerization. Polyphosphorylated phosphoinositides (PIPs) of parasite origin and their specific kinase are exported into the host cell cytosol and participate in its endocytosis. Hb delivered by endocytic vesicles to the FV releases its ferriprotoporphyrin IX (FPIX) and the globin is degraded by sequential action of aspartate proteases, cysteine proteinases, and dipeptidylaminopeptidase. The parasites does not have carboxypeptidase activity, and amino peptidase, distributed between the FV and the cytosol, terminate the digestion to individual amino acids. FPIX released from Hb during vacuolar digestion is a noxious compound that is detoxified by polymerizing it to hemozoin, assisted by histidine-rich proteins and lipids. A small portion of FPIX evades polymerization and is destroyed in the cytosol by GSH.

Other redundancies are exemplified by the generation of various mutants: clones with deletions in each of the individual PM I, PM II, and HAP genes as well as clones with a double PM IV/PM I disruption are viable and appear morphologically normal. PM II and PM IV/I disruptions have longer doubling times than the 3D7 parental line in rich RPMI medium. But in amino acid–limited medium, all four knockouts exhibit slower growth than the parental strain (Liu 2005; Bonilla 2007).

The biogenensis of falcipain 2 resembles that of plasmepsins (Dasaradhi 2007). Disruption of falcipain-1, falcipain-2, and falcipain-2′ was achieved. In each case, knockouts multiplied at the same rate as wild-type parasites. The morphologies of erythrocytic falcipain-1 and falcipain-2′ knockout parasites were indistinguishable from those of wild-type parasites (Sijwali 2006). The falcipain-3 gene could not be disrupted, indicating that it is essential for the parasite. The acquisition of external amino acids and Hb digestion are partially redundant nutrient supply routes, as is the redundancy of dual protease families with overlapping functional usage (Liu 2006).

As can be seen from Figure 9.36, Hb degradation is a gradual process, digesting full-length Hb chains with plasmepsins to large polypeptides and by means of falcipains, HAP, and falcilysin to small peptides. These are hydrolyzed by dipeptidyl aminopeptidase (EC 3.4.14.1), thus serving as a bridge between the endopeptidases and the aminopeptidases (Wang 2011). Two amino peptidases have been located to the food vacuole and demonstrably depend on acidic pH for their function: aminoacylproline- (EC 3.4.11.9) appears also in the cytosol and alanyl-aminopeptidase (EC 3.4.11.2). All other aminopeptidase are cytosolic, and much less is known about their function and essentiality.

Altogether, the parasite digests about 70% of the host cell cytosol (Hanssen 2011), but only 16% of the derived amino acids are used for protein synthesis (Krugliak 2002), and the major purpose of Hb digestion is the maintenance of host cell colloid osmotic pressure in view of changes in cation composition (Lew 2004; Mauritz 2011; Mauritz 2009).

A major waste product of Hb digestion is ferriprotoporphyrin IX (FPIX), which is demonstrably toxic for the parasite. To avoid intoxication, FPIX is biomineralized into hemozoin, the malaria pigment. This is a rather intricate process, which is abrogated by some antimalarial drugs, and readers are directed to a review for details (Egan 2008). In the present context, suffice to say that histidine-rich proteins (HRP) II and III (Sullivan Jr 1996; Lopez-Estrano 2003), lipids are involved in hemozoin formation (Pandey 2003). Both HRPs and HDP use the outbound–inbound trafficking route, like plasmepsins. Although most of the FPIX ends in hemozoin (Egan 2002), a part of which increases in the presence of chloroquine leaves the FV and is degraded by glutathione in the parasite cytosol (Zhang 1999). A heme detoxification protein has been identified that is shown to enhance heme-to-hemozoin transformation and to be essential for parasite life (Jani 2008).

Some reflections for the future

Much has been done in the last decades to unravel the special biochemistry of the malaria parasite. Although only *P. falciparum* has been discussed here, additional details were obtained from other *Plasmodium* species that are amenable to *in vivo* studies. The basic biochemistry of the parasite needs further probing to reveal potential drug targets, although the latter quest has not produced impressive progress since the end of the genome project of *P. falciparum*. An emphasis should be put on identifying the genes encoding missing enzymes, on metabolomics that has so far unraveled some

unexpected results such as glutamine/glutamate, providing most of the carbon skeletons of the TCA cycle intermediates, and on protein–protein interactions to probe the essence of enzyme and pathways regulations.

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CHAPTER 10
Signaling in malaria parasites

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Extracellular signals are received by receptors present at the cell surface, and the information encoded in these signals is transmitted inside the cell, its biochemical machinery eliciting biological responses. Given the importance of signaling in most cellular processes, the understanding of signaling pathways that operate in the malaria parasite will provide deeper insights into the molecular mechanisms involved in its development. The sequencing of the *Plasmodium* genome (Gardner 2002; Kissinger 2002) and subsequent bioinformatics and computational analyses indicated the presence of several interesting gene and protein families in the parasite (Aravind 2003; Ward 2004; Wilkes and Doerig 2008). One of the major highlights was the presence of diverse families of signaling proteins (Aravind 2003; Bahl 2003; PlasmoDB; Ward 2004; Wilkes 2008) in the parasite. A significant advancement in our understanding of parasite signaling events has been made in the last decade. In this chapter, some of the features of *Plasmodium* signaling regulated by second messengers like calcium, phosphoinositides, and cyclic nucleotides are discussed.

Protein phosphorylation in *Plasmodium*

Reversible phosphorylation of proteins, which is mediated by protein kinases and phosphatases, plays a pivotal role in signal transduction (Hanks and Hunter 1995). Significantly, phosphoproteomic studies indicate that almost 30% of parasite proteins are phosphorylated, highlighting the importance of phosphorylation in *Plasmodium* (Treeck 2011). The malaria parasite contains several eukaryotic protein kinases, which includes eukaryotic-like protein kinases (ePK) and atypical protein kinases (aPK). The kinome of human malaria parasite comprises about 90 protein kinases (PKs), which includes both ePKs and aPKs (Billker 2009; Ward 2004). In addition, *Plasmodium falciparum* contains a novel class of FIKK (family of protein kinases), which seem to be restricted to the apicomplexan parasites (Ward 2004). The bioinformatics studies suggested a lack of classical tyrosine kinases from the parasite (Ward 2004), although tyrosine phosphorylation of *Plasmodium* protein was observed (Solyakov 2011; Treeck 2011). Efforts to disrupt all protein kinase genes of *P. falciparum* and *Plasmodium berghei* (Solyakov 2011; Tewari 2010) suggested that a significant number of PKs are indispensable for the proliferation of blood-stage parasites (Solyakov 2011; Tewari 2010).

Although these studies highlighted the essentiality of several PKs, their specific role in parasite development could not be determined due the lack of an effective conditional gene knockout system. Approaches like the use of a FKBP death domain (DD) fusion, which destabilizes a protein

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in the absence of its ligand shield (Armstrong and Goldberg 2007; Russo 2009) and stage-specific gene disruption (Sebastian 2012) hold promise to study the function of essential genes. Although not essential for blood-stage development, several PKs emerged as key players in various stages of development in the mosquito vector and sexual differentiation of the parasite (Solyakov 2011; Tewari 2010). Pharmacological approaches have also been used to study the function of several of the essential PKs.

PKs control fundamental processes in the parasite. For instance, the parasite cell cycle may be tightly regulated by several CMGC and cell cycle–related kinases (reviewed in (Doerig 2008; Miranda-Saavedra 2012; Solyakov 2011; Tewari 2010)). Protein translation may be controlled by elongation initiation factor (eIF) kinases, which have been implicated in important processes like intraerythrocytic schizogony (Zhang 2012), latency in sporozoite development (Zhang 2010) and amino-acid sensing (Babbitt 2012).

The parasite has about 30 protein phosphatases designed to dephosphorylate parasite proteins (Wilkes 2008), which includes protein tyrosine phosphatases (PTP) (Andreeva and Kutuzov 2008). Compared to PKs, the information relating to the regulation and function of protein phosphatases is primitive. A report suggests that the gene disruption of a unique Kelch-domain containing phosphatase abrogates ookinete differentiation and motility (Guttery 2012; Philip 2012). Here, various *Plasmodium* protein kinases and other signaling proteins will be discussed in the context of the relevant signaling pathways.

Calcium-mediated signaling in *Plasmodium*

Calcium homeostasis and its role in the parasite life cycle

The oscillations in the levels of cytosolic free Ca^{2+} regulate diverse biological functions in eukaryotic cells. After influx from extracellular milieu, Ca^{2+} is stored in intracellular compartments and is released in response to various stimuli (Carafoli 1987). The malaria parasite and other apicomplexans like *Toxoplasma* require calcium for key processes like the secretion of adhesions, egress, motility, and invasion (Moreno and Docampo 2003; Moreno 2011). Typically, eukaryotic cells need millimolar amounts of extracellular Ca^{2+} to maintain intracellular concentration in the nanomolar range (Berridge 2003). During the blood-stage development, the concentration of resting cytosolic Ca^{2+} in the malaria parasite is also in the nanomolar range (Garcia 1996; Gazarini 2003). Intriguingly, the malaria parasite manages to survive in red blood cells (RBCs), which have low levels (~100 nM) of Ca^{2+} (Alleva and Kirk 2001). The Ca^{2+} entry into eukaryotic cells is facilitated by Ca^{2+} channels like the receptor-operated or voltage-gated Ca^{2+} channels. There is no concrete evidence that suggests the presence of these Ca^{2+} channels in *Plasmodium* (Moreno 2003, 2011). Therefore, the mechanisms via which Ca^{2+} enters the parasite are poorly understood.

The endoplasmic reticulum (ER) is the major store of Ca^{2+} in most eukaryotic cells. In addition, Ca^{2+} is stored in an acidic environment in acidocalciosomes and in the mitochondria. These stores of Ca^{2+} have been reported in the malaria parasite (Dluzewski and Garcia 1996; Garcia 1996; Moreno 2003). The influx of Ca^{2+} into the ER is mediated by sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), which transports Ca^{2+} in exchange for ATP hydrolysis. A SERCA-type calcium ATPase, PfATP6, is conserved in *Plasmodium* (Eckstein-Ludwig 2003), and thapsigargin, a specific inhibitor of SERCA-Ca^{2+} ATPase, triggers Ca^{2+} release in the parasite cytosol (Garcia 1996; Gazarini 2003). The release of Ca^{2+} from the intracellular stores is a tightly regulated process. Phospholipase C (PLC) acts on PI(4,5)P2, leading to the generation of I(1,4,5)P3 (IP3) and diacylglycerol (DAG), which are potent second messengers. IP3, in turn, interacts with the IP3-recptor (IP3R) on the ER and facilitates Ca^{2+} release.

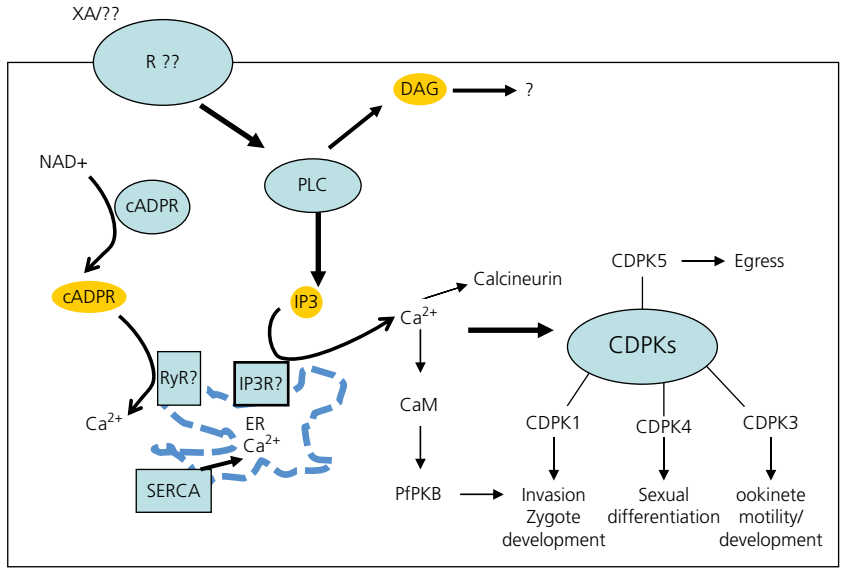

Figure 10.1 Calcium homeostasis and signaling in *Plasmodium*. Calcium is stored in the endoplasmic reticulum (ER), acidocalciosomes, or mitochondria (not indicated here) in the parasite. SERCA-like calcium ATPase may facilitate the entry of calcium into the ER. The activation of unidentified receptors (??) by extracellular signals like xanthurenic acid may cause the activation of phospholipase C PfPLC, which yields IP3 and DAG. IP3 facilitates the calcium release from the ER, which may also be mediated by the cADP–ribose–RyR–like pathway. The IP3R and RyR have not been identified in the parasite. Ca^{2+} regulates a wide variety of parasitic processes via targets like CDPKs, which play diverse roles in calcium signaling.

The parasite contains a phosphoinositide-specific phospholipase C (PfPLC), which can be inhibited by PLC inhibitor U73122. PfPLC is refractory to gene disruption and therefore is an essential enzyme for the asexual blood-stage parasites (Raabe 2011). Its inhibition by PLC inhibitor U73122 blocks several important parasitic processes such as host cell invasion and microgametocyte differentiation (Beraldo and Garcia 2005; Raabe 2011; Singh 2010; Vaid 2008). Ca^{2+} release from intracellular stores may also be mediated by cADP-ribose, which acts on RyR channels to trigger intracellular Ca^{2+} release (Figure 10.1). Pharmacological inhibition of this pathway also prevents the invasion of the host RBCs (Jones 2009), suggesting the possible existence of the cADP–ribose pathway in the parasite. It is important to note that both IP3R and RyR channels cannot be identified in the parasite based on sequence homology searches, suggesting that the parasitic receptors may be different from the host.

The modulation of the extracellular Ca^{2+} (McCallum-Deighton and Holder 1992) as well as the intracellular Ca^{2+} (Singh 2010; Vaid 2008) blocks invasion. The zoite forms of *Plasmodium* and other apicomplexan parasites contain specialized secretory organelles like rhoptries, micronemes, and dense granules, which release proteins that are relevant for the process of invasion (Cowman and Crabb 2006). The levels of extracellular potassium seem to regulate the free parasitic Ca^{2+} in the cytosol necessary for invasion and microneme secretion (Singh 2010; Singh and Chitnis 2012), although the underlying mechanisms need to be worked out. Ca^{2+} signaling has been implicated in the motility of tachyozoites, sporozoites, merozoites, and ookinetes (Billker 2009; Moreno 2011). In this context, its ability to mediate the phosphorylation of the components of glideosome assembly and actin–myosin motor complex may be relevant (Green 2008; Ridzuan 2012; Thomas 2012; Vaid 2008). PLC-mediated Ca^{2+} release regulates the phosphorylation of glidesome associated protein

(GAP45) in the merozoites (Thomas 2012). Xanthurenic acid is a mosquito-derived product of tryptophan catabolism that promotes the development of sexual stages of the parasite (Billker 1998, 2004). Xanthurenic acid triggers Ca^{2+} release in a PLC-dependent manner, which may induce differentiation of microgametocytes to microgametes (Raabe 2011).

Calcium-dependent protein kinases and other calcium effectors in malaria parasites

The release of Ca^{2+} from intracellular stores triggers downstream signaling events mediated by this second messenger. Ca^{2+} participates in diverse signaling events by interacting with effecter proteins. Typically, Ca^{2+} interacts with protein motifs and domains present on proteins, which include the EF-hand motifs and the C2 domain. Several *Plasmodium* proteins contain calcium-binding domains, which represent potent Ca^{2+} effectors. For example, close to 70 EF-hand–containing proteins are present in *Plasmodium* (Bahl 2003; www.plasmodb.org). Calmodulin, a well-known calcium-binding protein present in most eukaryotic cells, is expressed in *Plasmodium* (Cowman and Galatis 1991; Vaid and Sharma 2006). Calmodulin inhibitors stall parasite growth and block RBC invasion (Matsumoto 1987; Scheibel 1987; Vaid 2008). PfPKB, a protein kinase B–like enzyme (Kumar 2004), was shown to be a Ca^{2+}/calmodulin(CaM)–dependent kinase. Calmodulin interacts with PfPKB N-terminal region and stimulates its activity. CaM–PfPKB association was demonstrated in the parasite, and phospholipase C–mediated Ca^{2+} release promoted this interaction and subsequent activation of PfPKB (Vaid 2006, 2008). Although attempts to disrupt the PfPKB gene were suggestive of its essentiality for the asexual parasite, the inhibition of PfPKB by peptide or pharmacological inhibitors implicated it in RBC invasion (Vaid 2006, 2008). These studies resulted in the identification of a multicomponent signaling pathway that may be important for RBC invasion by the parasite (Vaid 2008).

Calcium-dependent protein kinases (CDPKs) are one of the major targets of Ca^{2+} in *Plasmodium*. CDPKs are absent from the host and are found in plants and protists (Harper and Harmon 2005). CDPKs possess an N-terminal kinase domain and a C-terminal four–EF-hand motif containing calmodulin-like domain (CLD), which are separated by a central regulatory domain. *Plasmodium* contains five CDPKs, which share this domain architecture (Tewari 2010; Ward 2004). In addition, two other kinases with EF-hand motifs with a distribution pattern different from CDPKs are also present in the parasite (Ward 2004). Biochemical studies on *Plasmodium* CDPKs, PfCDPK4, and PfCDPK1 suggested that the regulatory domain, which is also known as the J-domain, plays a pivotal role in the activation of CDPKs (Ahmed 2012; Ranjan 2009). The availability of crystal structures of *Toxoplasma gondii* CDPK1/3, which are closely related to PfCDPKs in the inactive and calcium-bound active form (Ojo 2010; Wernimont 2010), proved very useful for understanding the regulation of *Plasmodium* CDPKs. These structures and the homology model for PfCDPK1 revealed that in the inactive form, the CLD may interact with the kinase domain and the helical regulatory domain, which blocks the catalytic cleft and interacts with key residues of the kinase domain. Ca^{2+} binding to the CLD causes a major conformational change and rearrangement of domains. The CLD wraps around the regulatory domain, distorts it into three short helices, and moves away from the front of the kinase domain. Studies performed on PfCDPK1 suggested that the autophosphorylation of residues in various domains is critical for its activation, because it may stabilize key interactions (Ahmed 2012). Phosphoproteomic studies suggested that PfCDPK1 and other CDPKs may be phosphorylated at several sites in the parasite (Treeck 2011). Some of the CDPKs contain an N-terminal myristoylation signal, which in the case of PfCDPK1 was shown to target it to the membrane (Green 2008). Phospholipase C–mediated Ca^{2+} release regulated the activation of PfCDPK4 and PfCDPK1 (Ranjan 2009; Thomas 2012).

Attempts to disrupt PfCDPK1 were useful and suggested that it may be essential. Its pharmacological inhibition indicated that it may be involved in RBC invasion (Kato 2008). PfCDPK1 phosphorylates some of the proteins of the glideosome complex, which is needed for anchoring of the actin–myosin motor. It phosphorylates myosin A-tail interacting protein (MTIP) (Green 2008), the myosin light chain homologue, and PfGAP45 (Ridzuan 2012; Thomas 2012; Vaid 2008), which are key components of the glideosome (Keeley and Soldati 2004). Given the importance of the glideosome and the motor in motility and invasion (Keeley 2004), it is possible that one of the functions of PfCDPK1 may be in motor regulation, but further validation may be needed. PbCDPK1 was knocked down specifically in the sexual mosquito, which caused a developmental arrest and blocked mosquito transmission; it may translationally activate mRNA species in the zygote development, which otherwise remain repressed in macrogametocytes (Sebastian 2012). The function of CDPK1 in translation is surprisingly different from its proposed role in invasion (Kato 2008, Pushkar Sharma and Co-workers, unpublished).

Clearly, CDPK1 substrates need to be identified to gain insights into the mechanisms via which this kinase controls diverse parasitic processes. The gene deletion of PbCDPK4 suggested that it is not essential for asexual parasite growth. However, it is involved in promoting Ca^{2+}-mediated cell cycle progression of the male gametocyte (Billker 2004). Although parasitic substrates for CDPK4 have not been identified, it phosphorylates PfMap2 *in vitro,* which is an atypical kinase needed for the late-stage differentiation of male gametes (Tewari 2005). PfCDPK4 localizes to parasite periphery, and PLC-mediated Ca^{2+} release may regulate its activity in the parasite (Ranjan 2009).

Gene disruption of PbCDPK3 resulted in reduced gliding speed and transmission of ookinetes (Siden-Kiamos 2006). Unlike CDPK1, which has a myristoylation motif, CDPK3 is cytosolic in ookinetes. Therefore, these enzymes may have different substrates in the parasite. Although PfCDPK5 gene disruption was not possible, its knockdown mediated by using a DD domain fusion construct blocked the parasite's egress from the host RBCs (Dvorin 2010). The importance of various CDPKs in the parasite biology and their absence from the host has made them attractive antimalaria target (Kato 2008; Lucet 2012; Ojo 2012) (Figure 10.1). The parasite contains a calcineurin or protein phosphatase 2B (PP2B) homologue, which is regulated by Ca^{2+} and calmodulin and is inhibited by parasite cyclophilin A. This phosphatase may dephosphorylate proteins in response to calcium signaling (Dobson 1999).

Phosphoinositide signaling and trafficking in malaria parasites

Phosphoinositide metabolism in the parasite

The phosphorylation of 3′,4′, and/or 5′-OH of precursor phosphatidylinositol (PI) by various PI-kinases (Vanhaesebroeck 2001) leads to the generation of mono- or polyphosphorylated PIs (PIPs). One of the PIPs, $PI(4,5)P_2$, is hydrolyzed by phospholipase C (PLC) to $I(1,4,5)P_3$ and DAG. The phosphoinositides, IP3, and DAG also act as second messengers in most eukaryotic cells. Typically, PIPs participate in cellular signaling by interacting with specific protein domains and motifs (Lemmon and Ferguson 2001). PIPs are present at the plasma membrane and also on intracellular organelles such as the endosomes, vesicles, and Golgi (Gillooly 2000). Their interaction with domains like the FYVE, PH, and PX targets proteins on which these domains are present to subcellular locations in the cell relevant for the function of these target proteins (Gillooly 2001, 2003; Lemmon 2003).

Phosphoinositide analysis of the blood-stage malaria parasite suggested that several PIPs, which include PI3P, PI4P, PI(3,4)P2, and PI(4,5)P2, are synthesized in the parasite (Tawk 2010). In addition, the *Plasmodium* genome codes for several PI kinases, which supports the presence of these phosphoinositides in the parasite (Tawk 2010). PfPI3K is the only PI3K present in *P. falciparum*, and it

closely relates to class III PI3-kinases (PI3Ks) like yeast Vps34. PfPI3K immunoprecipitated from the parasite can catalyze the formation of PI3P, PI(3,4P)2, and PI(3,4,5)P3, suggesting that it is the likely source of these phosphoinositides in the parasite (Vaid 2010). PfPI3K exhibited vesicular localization in various parasitic compartments and the parasite membrane and surprisingly was found exported to the host RBCs (Vaid 2010). An increase in the levels of phosphoinositides in host RBC upon malarial infection has been reported (Elabbadi 1994). It is likely that the parasite-exported PfPI3K contributes to the PIP pool in infected RBCs.

A PI4P-5 kinase was identified in *Plasmodium*. This enzyme converts PI4P to PI4,5P2 by phosphorylating the 5′ hydroxyl of PI4P, and ADP ribosylation factor 1 (ARF1) stimulates the activity of this enzyme. Interestingly, a neuronal calcium sensor (NCS) domain, which the mammalian enzyme lacks, is present at the N-terminal domain of the *Plasmodium* kinase (Leber 2009). It is possible that the *Plasmodium* PI4P5K is involved in cross-talk between Ca^{2+} and phosphoinositides. A PI4-kinase is also conserved in *Plasmodium*. This enzyme (PlasmoDB ID: PFE0485w) complemented the function of yeast PI4K pik1 (Kruger 2010).

The role of phosphoinositides in the parasite life cycle

PI3K is essential for blood-stage parasite growth of *P. berghei* (Tawk 2010). Its pharmacological inhibition leads to the accumulation of hemoglobin-laden vesicles in *P. falciparum* and reduced hemoglobin content in the food vacuole, the site of hemoglobin degradation in the parasite (Vaid 2010). Consistent with this, an increase in undigested hemoglobin (Hb) was observed in PfPI3K-inhibited parasites. These findings suggested that PfPI3K may regulate hemoglobin trafficking in the parasite; however, the exact mechanism needs to be elucidated. *In silico* analyses have suggested that several PIP-binding domain–containing proteins are present in the parasite (Pushkar Sharma, unpublished results). A FYVE domain containing protein (FCP) from *P. falciparum* interacts with PI3P and localizes to the parasite food vacuole (McIntosh 2007). Using a FYVE domain probe, which interacts with PI3P, PI3P was found to be associated with food vacuole and the apicoplast membrane. Based on this, PI3P was implicated in the transport of proteins to these compartments (Tawk 2010) (Figure 10.2).

A host targeting signal (Hiller 2004), also known as the PEXEL motif (Marti 2004), which is present downstream of the N-terminal signal sequence, facilitates the export of proteins to the host RBC. The PEXEL is a five-amino-acid motif R/KxLxE/D/Q. An ER-resident protease, plasmepsin V, was implicated in the cleavage of the PEXEL motif prior to protein transport to the host (Boddey 2010; Russo 2010). Interestingly, the PEXEL motif interacts with PI3P in the ER lumen (Bhattacharjee 2012b). Subsequently, the ER-resident protease plasmepsin V may cleave the PEXEL motif from proteins, which may be exported in vesicles to the parasite membrane (PM)/parasitophorous vacuole membrane (PVM) and subsequently to the erythrocyte (Bhattacharjee 2012a).

Although these studies indicate diverse and important functions PIPs may play in the parasite (Figure 10.2), the understanding of metabolism of PIPs and their role in parasite signaling is rudimentary at this point.

Cyclic nucleotide signaling in the malaria parasite

Cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic-guanosine monophosphate (cGMP) are used by almost all organisms for cellular communication (Berman 2005; Baker 2012). cAMP and cGMP are generated in the cell by the activation of adenylyl cyclase (AC) and guanylyl cyclase (GC), respectively. Hydrolysis of cAMP and cGMP to adenosine or guanosine 5′-monophosphate is performed by cyclic nucleotide phosphodiesterase (PDE) enzymes. The

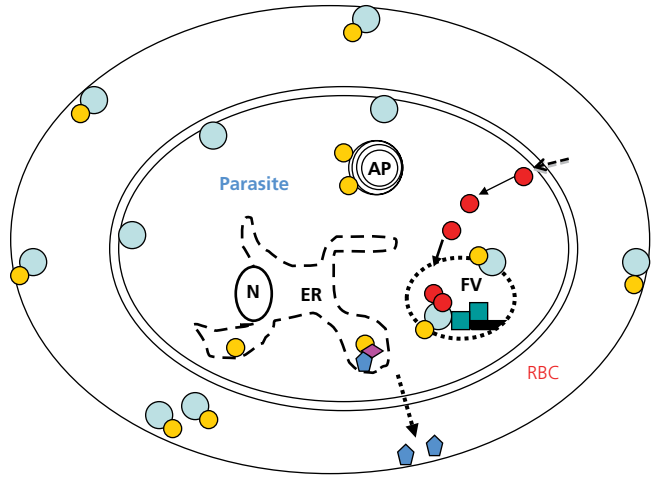

Figure 10.2 Phosphatidylinositol-3-phosphate (PI3P) in *Plasmodium falciparum*. *Plasmodium* codes for a single PI3-kinase, PfPI3K, which can generate PI3P (*yellow circles*) as well as other 3′-phosphorylated PIPs. PfPI3K (*light blue circles*) is present in vesicular compartments at PM/PVM and food vacuole. It is also exported to the host RBCs and it may generate 3′-PIPs at these locations. PI3P was localized at the cytoplasmic face of the food vacuole (FV) and apicoplast (AP) membrane and the endoplasmic reticulum (ER) lumen. It may be involved in the trafficking of proteins to these organelles. PfPI3K may regulate the endocytosis of hemoglobin (*red circles*) to the food vacuole. PI3P may interact with the PEXEL motif (*pink rhombus*) containing proteins (*blue pentagons*) in the ER, which facilitates their export to the host RBC after cleavage by plasmepsin V. PfPI3K exported to the host erythrocyte is active; however, its role in the host is unclear.

functions of cAMP and cGMP in the cells are performed by the effector serine/threonine kinases, such cAMP-dependent protein kinase (PKA) and cGMP-mediated protein kinase (PKG), respectively.

cAMP and cGMP generation in the parasite

The *Plasmodium* genome codes for the presence of two homologues each of ACs and GCs, which may be involved in the generation of cAMP and cGMP in the parasite (Bahl 2003; Gardner 2002; Moon 2009). PfACα has an N-terminal domain that shares features of a voltage-gated potassium-channel domain, which is not present in PfACβ (Muhia 2003). PfACβ has a putative dual catalytic domain with some features of a carbonate-sensitive prokaryotic AC (Baker 2011; Muhia 2003). While the catalytic activity for recombinant PfACα has been determined, PfACβ activity has remained elusive (Muhia 2003). The two PfGCs are bifunctional enzymes, with their C-terminus reminiscent of G-protein–dependent ACs. However, these enzymes generate cGMP and not cAMP (Baker 2004; Carucci 2000).

Four putative phosphodiesterase homologues are also present in the parasite; they share close to 40% identity at the amino acid level and also bear a class 1 signature motif (Baker 2011; Moon 2009). Whereas PDEα and PDEδ exhibit substrate specificity toward cGMP (Moon 2009; Taylor 2008), the specificity of the PDEβ and PDEγ has remained unknown.

Role of cAMP in parasite development

The finding that the parasite synthesizes its own cAMP was based on the activity of parasite adenylyl cyclase activity (Read and Mikkelsen 1991). cAMP signaling was suggested to be important for sexual commitment of the parasite because the addition of cAMP led to the transformation of asexual parasites to gametocytes (Kaushal 1980). In contrast to the host AC activity, forskolin or other

stimulators of G-proteins might not stimulate parasite AC (Read 1991), suggesting that parasite AC be different from the host enzyme and may operate independent of heterotrimeric G-proteins. This observation is supported by the proposed lack of classical heterotrimeric G-proteins from the parasite. It is important to note that the functional host G-protein–coupled β-adrenergic and adrenaline receptors and heterotrimeric G-proteins are present in the parasite-infected human RBCs (Harrison 2003). Although ACα is expressed at low levels in asexual parasites, gene disruption studies in *P. berghei* implicated it in the release of micronemal proteins from sporozoites (Ono 2008). Melatonin, a mammalian hormone that regulates circadian rhythm, was postulated to control the synchrony of malaria parasite development (Hotta 2000), which was dependent on PLC-mediated Ca^{2+} release. An intricate interplay between cAMP and calcium was also proposed in response to melatonin (Beraldo 2005).

PfPKA, a major effector of cAMP signaling in the parasite

Protein kinase A (PKA) is the major effector of cAMP signaling in cells. The interaction of cAMP with its regulatory subunit (PKA-R) results in its dissociation from the catalytic subunit (PKA-C), leading to the catalytic activation of the kinase. Both the subunits of PKA are conserved in the parasite (Haste 2012; Ward 2004) (Figure 10.3). Despite the sequence similarity, there are a few subtle differences in the catalytic subdomains of mammalian PKA-C and PfPKA-C. PfPKA-R shares significant homology with the core of mammalian PKA-R. However, their N-terminals are diverse: The PfPKA-R N-terminal has a putative myristoylation and palmitoylation signal (Haste 2012; Syin 2001; Wurtz 2011), which may target it to the parasite membrane. During asexual development, PfPKA is expressed in the schizont stages, with some expression in the ring and the trophozoite stages (Syin 2001). Gene- disruption studies suggest that PfPKA is essential for blood-stage development (Solyakov 2011), and its pharmacological inhibition by H89 and PKI stalled the parasite's growth (Syin 2001). It is suggested that the parasite forms new permeation pathways by altering the anion channel conductance in the host erythrocyte membrane. The overexpression of the PfPKA-R, which suppresses PKA-C activity in the absence of excess cAMP, led to the downregulation of whole-cell anion conductance, implicating PfPKA-R in host cell membrane conductance (Merckx 2008). PfPKA may have a role in the invasion of RBCs by the merozoite as it phosphorylates the C-terminal tail of AMA-1, a micronemal protein important for host invasion (Leykauf 2010). Clearly, additional PfPKA substrates need to be identified to understand its role in parasite biology.

cGMP in parasite development

The treatment of parasites with xanthurenic acid caused an increase in the activity of GC and cGMP production, leading to gametogenesis via PfPKG (Muhia 2001). In addition, xanthurenic acid–dependent synthesis of cGMP was inhibited upon addition of calcium (Muhia 2001) suggesting cross-talk between cGMP and calcium. Gene-disruption studies of GCβ in *P. falciparum* and *P. berghei* suggested that this enzyme might not be relevant for gametocyte or asexual blood-stage development of the parasite. In contrast, GCα turned out to be essential for asexual blood stages (Baker 2011; Hirai 2006; Moon 2009; Taylor 2008). Gene-disruption studies of various PbPDEs that hydrolyze cGMP have been attempted, and interesting phenotypes have been obtained (reviewed in Baker 2011).

PKG, a key regulator of cGMP-mediated signaling in the parasite

Protein kinase G is one of the major targets of cGMP and carries out signaling events in response to cGMP generation. *Plasmodium* contains a single PKG homologue (Ward 2004). PfPKG contains an N-terminal regulatory domain containing four cGMP binding sites and a C-terminal catalytic domain. Binding of cGMP to the N-terminal domain is necessary for the activation of the kinase

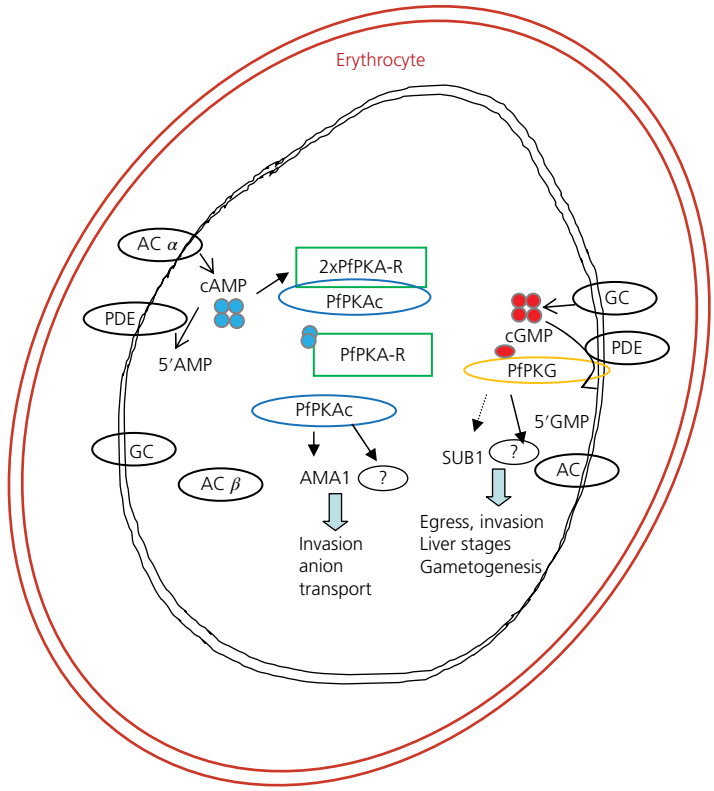

Figure 10.3 Cyclic nucleotide signaling in *Plasmodium falciparum*. Adenylate (AC) and guanylate cyclases (GC) generate cAMP and cGMP, respectively, and phosphodiesterases (PDE) hydrolyze these cyclic nucleotides. Binding of cAMP to PfPKA-R dissociates it from PfPKAc, resulting in its activation. PfPKG is activated upon binding of cGMP to its N-terminus. The identity of substrates for PfPKA and PfPKG will provide insights into their role in the parasite. Some of the parasitic processes in which these kinases may be involved are indicated.

(Deng and Baker 2002; Deng 2003). PKG gene disruption was not possible, and therefore it may be essential for asexual blood stages of *P. falciparum* (McRobert 2008; Moon 2009; Solyakov 2011). A conditional knockout strategy was used to delete a large portion of the PbPKG gene from liver-stage parasites, which caused a decrease in the merosome release (Falae 2010).

A pharmacological approach has been extremely useful in elucidating the function of PfPKG. Compound 1, a selective inhibitor of coccidian PKG (Gurnett 2002), inhibits PfPKG effectively (McRobert 2008). A mutant of PfPKG generated by replacing the gatekeeper residue in its ATP binding site (Shah and Shokat 2003) was significantly less sensitive to compound 1 (McRobert 2008). A parasite line was generated in which the wild-type PfPKG allele was replaced by the gatekeeper mutant. Using this parasite line, a role of PfPKG in asexual parasites, gametocyte development and liver-stage development was established (reviewed in Baker 2011). PfPKG may be involved in the early stage of development of male and female gametogenesis (McRobert 2008), and it may also be crucial for the rupture of blood-stage schizonts (Hopp 2012; Taylor 2010) (Figure 10.3).

PfSUB1 plays an important role in merozoite egress because it promotes the proteolysis of SERA and MSP proteins (Yeoh 2007). Compound 1 inhibition of PfPKG blocked MSP1 processing, suggesting that PfPKG might operate upstream of PfSUB1 (Dvorin 2010). As mentioned above,

defects in the late-stage schizont rupture of PfCDPK5 knockdown were also observed (Dvorin 2010). Whereas both PfCDPK5 knockdown and the inhibition of PfPKG arrested the schizont egress, the defects in invasion by merozoites were observed only in the case PfPKG inhibition. These observations suggested that CDPK5 may be a downstream effector of the egress-pathway or might operate independently. It will be interesting to probe the possibility of cross-talk between cGMP and calcium signaling in the parasites (Figure 10.3).

Future challenges

It is clear that almost all important stages of the parasite's development are regulated by cell-signaling cascades, and several key players, such as PKs, protein phosphatases, and cyclic nucleotide cyclases, involved in this process have emerged. There has been a significant advancement in our knowledge of *Plasmodium* signaling, which has shed light on novel aspects of parasite biology. However, major gaps in the understanding of parasite signaling pathways need to be filled. The upstream activators and downstream effectors of signaling molecules need to be identified, which would facilitate construction of signaling modules and aid dissection of the multicomponent signaling pathways in the parasite. There are hints of cross-talks between signaling mediated by different second messengers, which needs extensive evaluation and is likely to shed light on the feedback loops that might exert control on signaling.

Several signaling proteins are essential for the development of asexual blood stage parasite. Due to technical limitations, in most cases, their specific function in the parasite's biology remains unknown. Alternative approaches like the DD-domain–mediated degradation of proteins and chemical genetics, which have been used with some success, need to be refined further, and additional ones need to be developed. A multidisciplinary approach, which combines the traditional and the modern high-throughput approaches like genomics, proteomics, and chemical and systems biology will hopefully lead to the development of parasite-signaling maps, as is the case with other organisms.

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CHAPTER 11

Membrane transport proteins as therapeutic targets in malaria

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Malaria is leading cause of morbidity and mortality worldwide; estimates of lost productivity in endemic countries are also a call to action. In light of acquired resistance to most antimalarial drugs and a worrying delayed clearance phenotype with artemisinins, the current mainstay of treatment, new drugs with novel mechanisms of action are critically needed. The premiere agency for supporting discovery and development work, the Medicines for Malaria Venture (MMV), has a stated goal of taking a new drug through approval every 5 years (Anthony 2012). This is an ambitious goal based on the recognition that resistance, considered inevitable with most antimalarials, can quickly lead to a reversal of gains in public health.

A major direction in the past few years has been to identify libraries of anti-parasitic compounds by high-throughput *in vitro* parasite growth inhibition screens. These screens have generated thousands of hits with potentially useful activity against parasite proliferation, but the mechanism(s) of action are mostly unknown, making it difficult to prioritize compounds. Knowledge of the targets would provide key insights into the likelihood of acceptable *in vivo* activity, potential for acquired resistance, and risk of cross-reactivity with host activities. Most workers in this field believe that the pendulum will soon swing back to an emphasis on specific targets, or better yet, a balance between funding of these dichotomous approaches.

In this review, we explore membrane transport proteins of malaria parasites as therapeutic targets. Recent insights into the molecular basis and physiological roles served by some parasite-specific transport proteins suggest that greater support of drug discovery and development against these targets is warranted. There are several reasons that ion channels and transporters are especially attractive drug targets. First, comprehensive surveys of approved drugs and experimental agents for human disease reveal that about 15% of all existing therapeutics target transport proteins (Overington 2006), with a heavy emphasis on Na^+, K^+, and ligand-gated ion channels. This list includes a few anti-parasitic drugs. Most clearly, ivermectin, a favorite example of how big-pharma profit and global public health interests can sometimes be well-aligned, targets glutamate-gated chloride channels of helminths and has had a profound impact on reducing African river blindness caused by *Onchocerca volvulus* (Basanez 2006). Praziquantel, the first-line therapy for schistosomiasis

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and other helminths, also appears to work by effects on membrane permeability, but its precise mechanism of action is less well-established (Martin 1997).

Although a few antimalarial drugs have been proposed to act against parasite transport, the experimental evidence remains weak, and these proposals have not achieved general acclaim; we believe that parasite transport targets have been underexplored. Second, transport proteins are attractive targets because they serve critical roles in cellular homeostasis. Because of these roles, compounds that interfere with key transport processes often have profound effects on the cell. This may be especially important for blood stream malaria parasites, which move quickly between extracellular and intraerythrocytic sites, sense their environment through transmembrane signaling, import nutrients, and export soluble metabolic waste. Third, membrane transport proteins typically have more-constrained structures than soluble enzymes; this often improves the prospects for finding high-affinity inhibitors and can slow acquisition of resistance mutations.

Computational analysis of malaria parasite genomes reveals a paucity of conventional transport proteins with homology to transporters in higher organisms. Studies suggest that this is not because the parasite has fewer transporters than yeast or bacteria but rather because the parasite has evolved unique transport proteins that can only be identified through direct experimentation.

Rather than providing a comprehensive survey of all parasite-transport targets, we have chosen to focus our review on specific membrane transport proteins that we find particularly attractive. Each of us selected one or more targets from the malaria research literature based on the evidence for transport activity, the level of molecular characterization, the understanding of the physiological role served, and the availability of methods for developing specific therapies. We emphasize transporters expressed primarily in asexual blood stream stages, which are both the best studied and the most significant clinically. There are clearly other transport proteins in malaria parasites that may be good therapeutic targets.

These targets are presented as separate sections based on the membrane where they reside (Figure 11.1A), starting from host plasma and moving inward to compartments within the intracellular parasite.

Host erythrocyte membrane: A shared ion and nutrient channel

The permeability of the host erythrocyte membrane increases dramatically after infection. These changes are easily detected and, in fact, form the basis of methods that use sorbitol to synchronize *in vitro* parasite cultures and to separate infected erythrocytes from uninfected cells by density gradient centrifugation. Because the host membrane is readily accessible to research, transport at this membrane has been better studied than at any other membrane in the parasite's complex life cycle. These studies span some 7 decades and have used diverse methods – flamephotometry of *ex vivo* infected cells, tracer flux, osmotic fragility, fluorescence-based assays, and various configurations of patch-clamp – to characterize the magnitude and biophysical properties of this increased permeability (Overman 1948; Kutner 1982; Kirk and Horner 1995; Desai 2000; Staines 2007). These studies have determined that inorganic anions as well as organic solutes of varying size, polarity, and charge have markedly increased permeability after infection; inorganic cations have more modest increases, with Na^+ better excluded than K^+ (Ginsburg and Stein 1988; Staines 2000; Cohn 2003; Bokhari 2008).

Although there may be contributions from upregulated host membrane transporters (Staines 2007), uptake of most solutes after infection can now be attributed to a single broad permeability ion channel known as the plasmodial surface anion channel (PSAC) (Alkhalil 2004). This channel is an attractive drug target for several reasons.

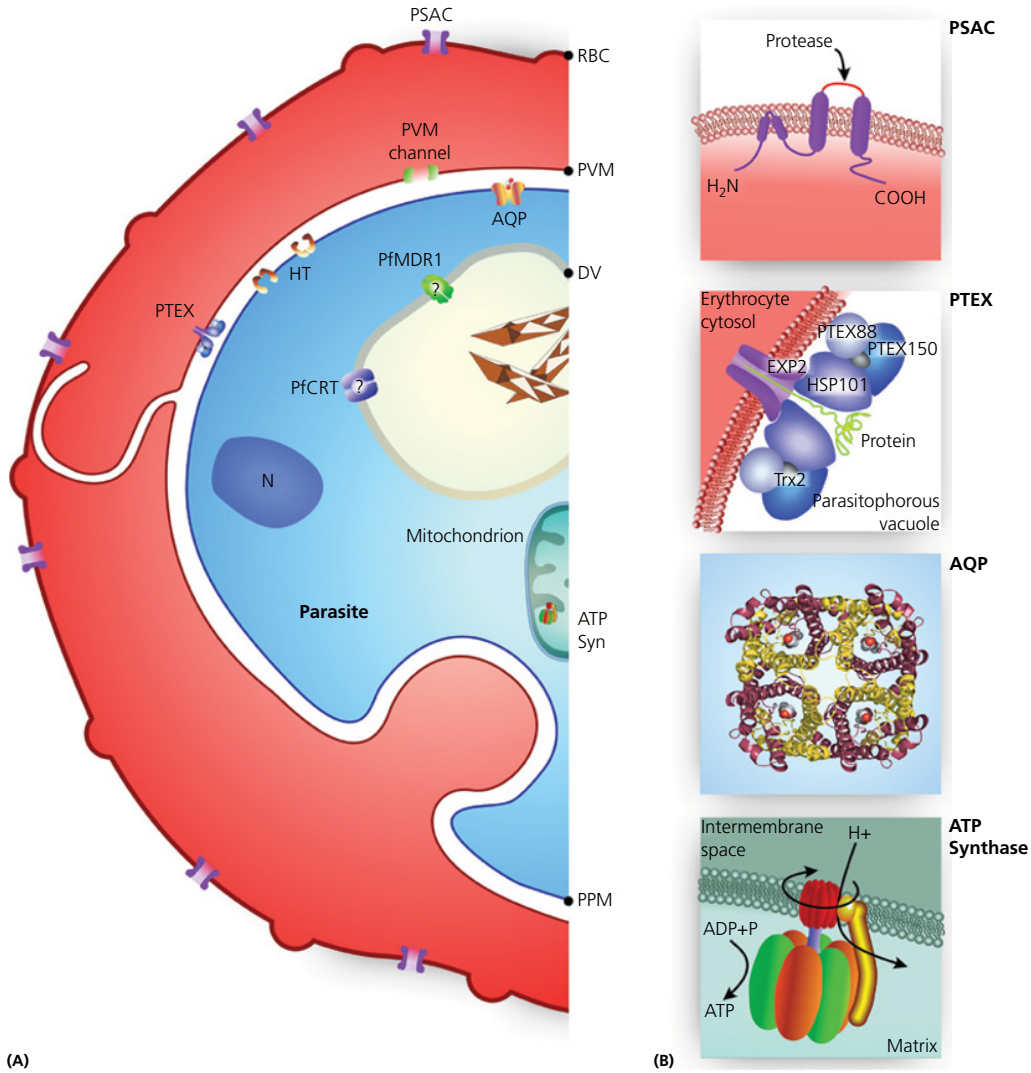

Figure 11.1 *A,* Schematic showing the multiple membranes within an infected erythrocyte and locations of specific transport proteins. PSAC is on the host erythrocyte membrane (RBC). PTEX and the PVM channel reside on the parasitophorous vacuolar membrane (PVM). A glucose transporter (HT) and an aquaglyceroporin (AQP) are shown on the parasite plasma membrane (PPM). PfCRT and PfMDR1 are shown on the digestive vacuolar membrane (DV); question marks reflect uncertainties in the properties of these transporters. Hemozoin is shown as crystalline material within the DV. ATP synthase (ATP Syn) localizes to the inner membrane of the mitochondrion. N indicates the nucleus. *B,* Individual panels showing relevant features of selected transporters. PSAC activity is determined by CLAG3, a protein that is integral to the host membrane; purple ribbon diagram shows a possible transmembrane topology of CLAG3. A highly variable domain (shown in red) is exposed at the host membrane, based on protease susceptibility studies (Nguitragool 2011). PTEX localizes to the PVM membrane, is a high-molecular-weight complex of several parasite proteins, and may function as a protein translocon; adapted from de Koning-Ward 2009, with permission. Crystal structure of AQP from *P. falciparum*, showing four-fold symmetry and four parallel routes for water transport; adapted from Newby 2008 with permission. ATP synthase is a multi-subunit motor on the inner mitochondrial membrane; in most organisms, this enzyme uses the downhill uptake of H^+ into the matrix to synthesize ATP. In a few organisms, H^+ is exported using the energy of ATP hydrolysis; the precise function in malaria parasites is uncertain.

First, this transport activity is strictly conserved in malaria parasites: the list of permeant solutes, their relative permeabilities, and pharmacology are conserved not only among divergent lines of the human pathogen *P. falciparum* but also among all examined malaria species. Indeed, patch-clamp studies of *Plasmodium knowlesi*–infected rhesus monkey erythrocytes yielded single channel activity with gating and conductance properties almost indistinguishable from those seen with *P. falciparum*–infected human erythrocytes (Lisk and Desai 2005). Paralleling this functional conservation, the *clag3* genes are also conserved across this genus. At the same time, neither these genes nor similar channel activity are present in *Babesia*, related parasites that also infect erythrocytes (Alkhalil 2007); similar channels are also unknown in higher organisms.

Second, the surface location on infected cells renders PSAC readily accessible to soluble drugs in host plasma, reducing drug design constraints on membrane permeability and molecular weight. This location is also not susceptible to acquired resistance via extrusion of drug from the site of action, a common mechanism in malaria as described in a later section.

The third advantage of PSAC as a drug target is the collection of robust methods for quantifying its activity; both high-throughput screens for potent inhibitors and inhibitor mechanism-of-action studies on single-channel molecules have been carried out (Pillai 2010).

An important debate in this field has focused on the number of distinct ion channels at the host membrane and their relative contributions to increased permeability. This debate is central to determining the prospects for drug discovery: redundant pathways for nutrient uptake would presumably allow parasites to evade killing by specific inhibitors of individual channels. Much of the uncertainty has arisen because of differing patch-clamp results in separate labs (Verloo 2004; Staines 2007; Bouyer 2011; Kucherenko 2012); these inconsistencies may reflect key differences in experimental procedures (Lisk and Desai 2006), as reviewed by Desai (2012). We believe that this uncertainty has now been largely resolved because several lines of evidence strongly suggest a primary role for PSAC in the increased permeability of most small solutes at the host membrane of infected cells.

First, inhibitor studies have revealed quantitative correlations in dose-response studies with multiple solutes. Regardless of chemical scaffold or affinity, multiple inhibitors have yielded constant activity against uptake of sugars, amino acids, organic cations, and inorganic anions (Alkhalil 2004; Kang 2005; Desai 2005; Pillai 2010). For each inhibitor, uniform affinity for PSAC block has been quantitatively observed with macroscopic methods (*e.g.,* tracer flux and osmotic fragility) and with single channel patch-clamp, with an important caveat relating to solute-inhibitor interactions (Lisk 2007). These observations suggest a single shared ion channel because solutes transported by unrelated ion channels invariably exhibit differences in pharmacology.

The second line of evidence relates to transport mutants generated by *in vitro* selections with blasticidin S and/or leupeptin (Hill 2007; Lisk 2008; Lisk 2010), toxins that require PSAC-mediated uptake to reach their intracellular targets. *In vitro* selections with these toxins yielded transport mutants, presumably by favoring altered channels that reduce toxin uptake at the host membrane. Because each mutant was found to exhibit parallel changes in organic solute uptake and channel behavior in single PSAC recordings, these findings provide independent evidence for a single shared ion channel.

Maybe most importantly, a high-throughput screen has identified a remarkably specific small molecule inhibitor known as ISPA-28 (an acronym for *i*solate-*s*pecific *P*SAC *a*ntagonist). This compound has about 800-fold greater affinity for inhibition of channels from cells infected with the Dd2 parasite line than from the HB3 line (Nguitragool 2011). This specificity directly addresses concerns about inhibitor promiscuity that plagued earlier studies. Demonstrated inactivity against the same channels, but from HB3 parasites, combined with predictable effects in allelic exchange transfections, indicate a defined inhibitor binding pocket on a single type of ion channel. Indeed, this remarkable inhibitor specificity was essential for the identification of parasite *clag3* genes as determinants of PSAC (Figure 11.1B) (Nguitragool 2011).

Despite these observations, contributions from other transporters on the host membrane cannot be unambiguously excluded. Some transporters have been reported to depend on parasite metabolic or enzymatic status, which can vary from lab to lab and may be influenced by *in vitro* cultivation conditions. Even with the evidence supporting a primary role for PSAC, the precise role of the *clag3* product in channel formation is unclear. In one scenario, the *clag3* product may need to associate with other polypeptide subunits, of host or parasite origin, to form the nutrient channel at the host membrane. Definitive resolution of this uncertainty may soon be possible through heterologous expression of *clag3* and other candidate genes and/or through transfection studies in human and rodent malaria systems.

How might PSAC inhibitors work in the treatment of malaria? A significant correlation between the potency of PSAC inhibitors and *in vitro* growth inhibition implicates an essential role for this channel (Pillai 2010), but the precise function has been debated. We recently addressed this unknown with modified *in vitro* culture conditions to determine that one essential role of the channel is in nutrient uptake at the host membrane (Pillai 2012). Because standard culture media contain supraphysiological levels of nutrients, we reduced the concentrations of three key solutes (isoleucine, glutamine, and hypoxanthine) to more physiological values and found marked improvements in parasite killing by PSAC inhibitors. At the same time, three existing antimalarial drugs that work at other parasite targets exhibited unchanged efficacies in our modified medium, excluding nonspecific effects of the engineered medium. Additionally, parasite killing by PSAC inhibitors under these conditions was successfully used to select for expression switching between *clag3* alleles and for rare parasites that carry an extra copy of *clag3.* An alternative proposed role has been that the increased permeability facilitates remodeling of erythrocyte cytosolic cation concentrations, possibly to benefit the intracellular parasite (Mauritz 2011). A study has confirmed that these changes occur because of PSAC-mediated cation transport, but it determined that they are not essential for parasite growth (Pillai 2013).

Drug discovery and development against PSAC is supported by conservation of the *clag3* genes and PSAC activity in all malaria parasites. Though considered a liability by some, the fact that *P. falciparum* has two copies of the *clag3* gene and invests in expression switching to facilitate immune evasion is further evidence of the channel's physiological importance to blood stream parasites. There remain some key unknowns that need to be addressed for continued progress toward a drug against this target.

First, it is important to define the precise role(s) served by PSAC under *in vivo* conditions, which may not be well-reflected by experiments using parasites from *in vitro* cultures. Second, determining exactly how *clag3* product contributes to channel formation as well as the identification of other possible subunits or interacting proteins is critical. Full knowledge of the channel's composition and structure should reveal the most druggable sites on the channel and may provide insights into the likelihood of acquired resistance. Third, demonstrated *in vivo* efficacy of PSAC inhibitors in rodent malaria models will be necessary to stimulate next-level investment by funding agencies and potential pharmaceutical partners. With new insights resulting from the identification of *clag3* genes as key determinants, there is optimism that definitive answers to these questions may soon be obtained.

Parasitophorous vacuolar membrane: Protein export and solute uptake

During erythrocyte invasion, the parasite surrounds itself with an extra lipid bilayer known as the parasitophorous vacuolar membrane (PVM). Although this membrane is originally derived from erythrocyte lipid (Ward 1993), the PVM surface area continues to increase with the maturing

intracellular parasite. Parasite lipids and proteins are added to this membrane, some at the time of invasion through packaging within organelles of the invasive merozoite and some at later time points (Spielmann 2012; Bullen 2012). It is unclear why malaria parasites retain their PVM while some other apicomplexan parasites have evolved ways to eliminate this barrier and enjoy direct access to the host cell cytosol.

Relatively little is known about the functions of most proteins at this membrane, but two important transport activities are clear: protein export and solute exchange.

Protein export

Many proteins manufactured in the parasite are exported to the host cell compartment, where they modify erythrocyte ultrastructure (Tilley and Hanssen 2008), permeability (Desai 2000), deformability (Mills 2007), cytoadherence (Chen 2000), and possibly other properties. Given the extent of host cell remodeling and the possibility of yet-unexplored roles, it is not surprising to find that some 300 to 400 parasite proteins carry the PEXEL/VTS signal that directs proteins for export (Marti 2004; Hiller 2004). Although both the parasite plasma membrane and the PVM are barriers to this export, canonical secretory machinery shared by all eukaryotic cells presumably allows passage of the parasite plasma membrane; transit across the PVM requires a discrete transmembrane transport mechanism. Although some workers distinguish this process from conventional channels and transporters that facilitate transmembrane movement of small solutes, the similarities—specific recognition of soluble substrate, movement of the substrate across a lipid bilayer, and unfavorable energetics without the involvement of a membrane embedded transporter are fundamental in nature. We therefore consider protein export at the PVM a bona fide transport target for antimalarial development.

A novel candidate for this PVM protein translocon has been proposed (de Koning-Ward 2009). This group used computational analyses to identify a stage-specific putative ATPase known as HSP101 based on the assumption that protein translocation across membranes requires energy to unfold and thread complex polypeptides through a translocon. They then employed a classical biochemical strategy to find interacting proteins and proposed a putative translocon complex of 5 proteins (Figure 11.1B). Antibodies against either specific subunits or against epitope tags added to individual components through DNA transfection have confirmed a high-molecular-weight complex that localizes to the PVM (Bullen 2012). Maybe the most compelling evidence for a role of this complex is that co-immunoprecipitation experiments demonstrate that the PTEX complex interacts with exported parasite proteins carrying the PEXEL motif. Reverse pull-down experiments found that exported proteins also precipitate PTEX components, allaying concerns of artifacts that often plague mass spectrophotometric studies of parasite cell lysates. Additional controls using PEXEL-linked GFP reporter constructs have provided additional supporting evidence.

One member of the PTEX complex, EXP2, forms a homo-oligomer refractory to extraction by Na_2CO_3 (Bullen 2012), indicating an integral membrane protein and suggesting that it may define an aqueous pore for passage of polypeptides through the PVM. Another component, HSP101, has an AAA^+ ATPase domain and homology to chaperone proteins known to contribute to protein translocons in other systems; based on this homology, this member may serve a role in unfolding proteins in the vacuole prior to export.

Although identification of the PTEX complex represents an important advance in malaria research, direct evidence for a role of the complex in protein export is still missing. The complex certainly appears to be a good candidate, but developing a straightforward functional readout for protein export should be a top priority in this field. Currently, this has been limited to visualization of GFP-tagged reporter proteins in mature infected cells, providing insufficient temporal and spatial resolution to directly link the PTEX complex to the export event. As protein translocons from other

systems have been successfully reconstituted into artificial membranes (Simon and Blobel 1991), there is cause for optimism in the malaria-transport field.

A number of other important questions need to be addressed. For example, it is unclear how exported proteins are recognized by the translocon. One possibility, direct recognition of the PEXEL export sequence by the translocon, is complicated by specific proteolysis within this export sequence prior to reaching the translocon (Chang 2008); the two-residue N-terminus of the cleaved protein (xE/D/Q) is N-acetylated and may be recognized by the translocon, but experiments suggest that this may not be sufficient (Boddey 2009). Some exported proteins, known as PNEPs, lack the PEXEL motif (Spielmann and Gilberger 2010); it is unclear what targets these proteins for export and whether they use the same translocon to cross the PVM. Another important question relates to whether chaperone proteins interact with exported proteins prior to, during, and after movement through the PVM translocon; identification and mechanistic studies of such interactions could help define the best points of attack. Finally, it will be important to examine if both soluble and membrane proteins, whether retained at the PVM or trafficked on to membranes in the erythrocyte cytosol, share a single translocon at the PVM.

A key hurdle in exploring therapies that target the PVM translocon is, as described above, the development of a functional assay for protein export. Development of such an assay should allow screening for specific inhibitors, of which there are none known currently. Based on the number of parasite activities that depend on export and function of proteins to the host compartment, such inhibitors are predicted to have lethal effects on both *in vitro* and *in vivo* propagation of the intracellular parasite.

Solute exchange

Our understanding of ion and small solute flux at the PVM is limited to two studies that used electrophysiological methods to identify and characterize a single large conductance ion channel on this membrane (Desai 1993; Desai and Rosenberg 1997). Quantifying solute transport at this membrane is complicated by the PVM's intracellular location, the close apposition to the underlying parasite plasma membrane, and lack of protocols for selective enrichment of this membrane from infected cells. Electrophysiological approaches are well suited for overcoming these limitations because they allow the isolated study of individual ion channel molecules on a chosen membrane.

The two studies suggest that the PVM channel is present at high density on the vacuolar membrane and that it is primarily open at the resting membrane potential, which together implicate high-capacity exchange between the erythrocyte and vacuolar compartments. The channel is indiscriminately permeant to cations, anions, and organic solutes up to 1200 daltons in size. One study used patch-clamp of individual trophozoite-infected cells and two separate strategies to expose the PVM for access by a patch-clamp pipette (Desai 1993); the other used functional reconstitution of the channel into artificial membranes, allowing manipulation of solute concentrations on both channel faces (Desai and Rosenberg 1997). Reassuringly, the channel's key biophysical properties – conductance rates in various media, gating properties, and voltage dependence – were nearly identical in these divergent studies. Based on the high copy number of this channel and its large measured pore size of 2.3 nm (Desai and Rosenberg 1997), one would guess that the parasite has little need for additional solute transporters or channels at this membrane.

Key hurdles in advancing this channel into the therapeutic pipeline include identification of the responsible genes, examination of the channel's physiological role, and development of methods for inhibitor screening. Advances in high-throughput electrophysiology of small cells should be enabling. Although there are many unknowns, the hypothesis that this channel may be the primary solute transport mechanism on the PVM should be strong motivation for additional fundamental studies.

Parasite plasma membrane: Similar to other eukaryotic cells, but different

The next membrane encountered, the parasite plasma membrane, has been actively studied by several groups. Much of this research has been facilitated by computational analyses of the parasite genome, which revealed orthologues of several transporters that localize to the plasma membranes of bacteria or higher eukaryotes. As might have been predicted, biochemical studies have determined that some of the plasmodial orthologues localize to the parasite plasma membrane (Figure 11.1A) (Rager 2001; Woodrow 1999).

Although homology to known host transporters may initially decrease motivation for drug targets on this membrane, transport at the parasite plasma membrane differs from that of host cells in several important ways; these differences should motivate workers to pursue therapeutic targets on this membrane. First, the parasite genome lacks a clear orthologue of the Na^+/K^+-ATPase pump, which is used by higher organisms to maintain desired intracellular concentrations of these cations. Na^+ and K^+ are the most abundant cations in both the host and the parasite; moreover, they serve numerous critical functions within cells as they modulate the activity of various enzymes, contribute to the electrical potential at many membranes, and drive the movement of other solutes through co-transport systems. Therefore, it seems likely that the parasite has one or more novel transporters, possibly ATPase pumps, for the regulation of these cations at the parasite plasma membrane. The transport of H^+ and Cl^-, two other important ions, is also likely to be quite different from that of higher organisms. Parasite plasma membrane transport mechanisms specific to these four ions deserve especially rigorous study in the future.

Although multiple transporters at the parasite plasma membrane have been reported and characterized to varying extents, we have chosen two for further discussion based on the level of study and the potential for development of therapies.

Aquaglyceroporin

Computational analyses of the parasite genome reveal very few bona fide ion channel genes. Of these, maybe the best studied is an atypical aquaglyceroporin gene with homology to water channels in cells ranging from bacteria up to humans. Peter Agre received the Nobel prize for discovering aquaporins and for uncovering, through numerous collaborative studies, the roles these channels serve in key physiological processes, the mechanism of water permeation without proton flux, and diseases resulting from mutations in the human channel genes.

In contrast to higher eukaryotes, plasmodia have a single aquaporin gene, PfAQP, which belongs to a subfamily permeant to both water and glycerol, the aquaglyceroporins (Hansen 2002). The encoded protein localizes primarily to the parasite plasma membrane and carries atypical mutations in the canonical NPA motifs that line the channel pore. Functional channels have been expressed in *Xenopus* oocytes, yeast, and *Escherichia coli* (Bahamontes-Rosa 2007; Hansen 2002; Newby 2008). Heterologous expression has revealed that PfAQP has a broader than usual solute selectivity profile, with substantial permeability of urea, 5-carbon sugar alcohols, ammonia, and various polar toxins (Hansen 2002). Heterologous expression has also permitted examination of critical residues in the channel protein through mutagenesis (Beitz 2004) and determination of the channel's crystal structure (Figure 11.1B) (Newby 2008).

While these studies provide an enviable level of insight into the workings of PfAQP, some critical questions remain. Maybe most importantly, the precise roles served by this channel are not well understood. Proposals include reducing parasite osmotic stress (*e.g.,* if the host becomes dehydrated from malaria or upon passage of infected cells through the kidney circulation), allowing uptake of

glycerol for synthesis of parasite phospholipids, and removal of parasite metabolites such as ammonia (Hansen 2002; Zeuthen 2006). These and other possible roles have been examined through DNA transfection, which has yielded a viable knockout line in *Plasmodium berghei* (Promeneur 2007). Mouse erythrocytes infected with the knockout line lack the high [^{14}C]glycerol uptake characteristic of wild-type infected cells. Infections in mice with this knockout line yielded lower parasitemias and increased survival of animals, indicating that PbAQP may function to increase *in vivo* parasite virulence. Although viability of the knock-out may be discouraging for drug development, whether a knock-out can be made in human parasites remains to be determined. Controlled infections in laboratory animals might also not be representative of stressors on the parasite in clinical malaria.

Only a few relatively nonspecific inhibitors are available for aquaporins in any system; their effects on parasite aquaglyceroporins have not been fully examined. Selective inhibitors that do not interfere with host aquaporins or other essential activities must be found if this well-studied target is to be advanced into the drug discovery pipeline.

Hexose transporter

Blood-stage parasites produce ATP primarily though anaerobic glycolysis. Because this is relatively inefficient, they must take up and consume larger amounts of glucose than host cells of comparable metabolic activity. Glucose enters infected erythrocytes through both PSAC and a host glucose transporter known as GLUT1 (Landfear 2010), diffuses through the PVM channel, and crosses the parasite plasma membrane primarily via a single conserved transporter known as PfHT. Starting from early heterologous expression studies using total *P. falciparum* mRNA injected into *Xenopus* oocytes, PfHT was identified as a close orthologue of GLUT1 in the early releases of the parasite genome sequence (Woodrow 1999). Cloning and microinjection of *pfht* cRNA into oocytes yielded increased uptake of D-glucose and 2′-deoxy-D-glucose in a stereospecific manner. D-glucose uptake exhibited higher affinity than that of the host GLUT1, as might be desirable for an intracellular parasite with reduced access to plasma nutrients. Uptake was saturable and Na^+ independent, suggesting a carrier-type mechanism that transports glucose alone without obligate coupling to other solutes.

A small survey of *O*-derivatives of glucose then identified 3-*O*-((undec-10-en)yl)-D-glucose (also known as compound 3361) as a competitive inhibitor of PfHT (Joet 2003). This compound is reasonably selective for the parasite transporter over the human GLUT1 transporter (K_i values of 53 μM and 3.3 mM, respectively). It inhibits *in vitro* parasite growth with an IC_{50} of 15 μM in standard medium, but efficacy is improved, as might be predicted, when external glucose is reduced. The compound has similar activity against glucose transporter orthologues in other malaria species and has been shown to inhibit *in vivo P. berghei* growth in mice (40% reduction in parasitemia using a four-day suppression test), a remarkable finding for a relatively low affinity competitive inhibitor. Transfection studies also appear to support this target because attempts to disrupt this transporter have been unsuccessful in both *P. falciparum* and *P. berghei* (Slavic 2010). Notably, disruption in *P. falciparum* could be achieved when PfHT was expressed from an episomal copy to allow functional complementation.

These studies provide proof of concept for targeting parasite glucose acquisition; expression of the protein in hepatic and mosquito stages suggests that inhibitors may also be effective in interfering with malaria transmission (Slavic 2011). We see two related hurdles that must be overcome to advance this antimalarial target. First, PfHT has homology to host glucose transporters, of which humans carry 14 distinct copies (SLC2A or GLUT protein family) (Thorens and Mueckler 2010). Can an inhibitor that is sufficiently specific for the parasite orthologue be identified? The second hurdle relates to obtaining inhibitors of sufficient potency to be clinically useful. Presumably, such

compounds will need to be noncompetitive and avoid the glucose scaffold. Fortunately, two groups have developed cell-based assays for high-throughput screening of inhibitors (Feistel 2008; Blume 2011). Because these assays use heterologous expression of either parasite or host glucose transporters to complement defects in glucose uptake, there is optimism that potent inhibitors specific for the parasite transporter may yet be identified.

Digestive vacuole: A specialized lysosome-equivalent in the parasite

An important intracellular compartment within the blood stream parasite is the digestive vacuole (DV), which resembles eukaryotic lysosomes because its contents are acidic and contain many digestive enzymes such as proteases and acid hydrolases. A critical activity in this compartment is digestion of hemoglobin, brought into the parasite through endocytosis of erythrocyte cytosol as an amino acid source for synthesis of parasite proteins (Francis 1997). Heme, also liberated by hemoglobin digestion, is detoxified within the digestive vacuole through enzymatic polymerization into hemozoin (Egan 2002). The high catabolic activity of the DV makes it susceptible to chemotherapeutic intervention, with chloroquine and a number of other approved antimalarials thought to work at this site (Slater and Cerami 1992; Jacobs 1987; Dong and Vennerstrom 2003). The DV membrane contains several putative transport proteins that appear to be critical for its function; two, PfCRT and PfMDR1 (*Plasmodium falciparum* chloroquine resistance transporter and multidrug-resistance transporter), have been linked to acquired antimalarial drug resistance and are considered here as drug targets.

Chloroquine (CQ), once a remarkably effective first-line treatment for human malaria, accumulates passively in the digestive vacuole (Aikawa 1972; Yayon 1984), where the acidic pH leads to protonation and trapping of a primarily di-protonated form of the drug (CQ^{2+}). Although the antiparasitic mechanism of CQ is not fully established, the drug is known to interfere with heme crystallization (Fitch, 2004). Acquired CQ resistance in malaria is characterized by reduced CQ accumulation in the DV. Importantly, the initial rates of radiolabeled CQ uptake are identical in erythrocytes infected with sensitive or resistant parasites, but initial efflux rates are about 50 times higher in resistant parasites, strongly suggesting transporter-mediated removal of CQ from the digestive vacuole as the mechanism of CQ resistance (Krogstad 1987). Several Ca^{2+} channel blockers and anticancer agents were found to function as chemosensitizers, retarding CQ efflux and restoring accumulation in resistant cells; these compounds had little or no effect on sensitive parasites. These early findings suggested a transport mechanism for CQ at the DV membrane, possibly one with homology to multidrug-resistance pumps identified in cancer cells.

Mutations in PfCRT largely determine whether treatment of malaria with CQ results in success or failure. This novel transporter was identified though an extended effort with a laboratory genetic cross between CQ-sensitive and -resistant parasite lines and confirmed with DNA transfection and studies of clinical isolates from malaria patients (Wellems 1991; Sidhu 2002; Djimde 2001). The gene product has ten predicted transmembrane domains and has been localized to the DV membrane. DNA transfection experiments have identified domains involved in trafficking and insertion at the DV membrane and have determined that the C-terminus is located on the cytoplasmic face of the DV membrane (Kuhn 2010). This membrane topology places a critical mutable residue, 76 (lysine or threonine in CQ-sensitive and -resistant parasites, respectively), near the end of the first predicted transmembrane domain and facing the DV lumen. Mutations in PfCRT linked to CQ resistance do not affect trafficking, distribution, or stability of the protein at the DV membrane (Ehlgen 2012). These findings suggest that the charge-changing K76T mutation contributes to an altered

substrate binding site on the putative transporter; the resulting change in solute transport may then produce acquired CQ resistance.

The other DV transporter we consider here, PfMDR1, was first identified through PCR amplification of the parasite's *mdr1* gene using primers that recognize conserved domains on *mdr* genes of higher organisms, with adjustment for the parasite's codon use (Foote 1989; Wilson 1989); these studies were partially motivated by the similarities between CQ-resistance and cancer chemotherapy–resistance phenotypes: Both involve drug efflux and are partially reversed by Ca^{2+} channel blockers. Because these studies found that that the *pfmdr1* gene was present in higher copy number in some chloroquine-resistant strains, they provided early circumstantial evidence to support a role of this putative transporter. This hypothesis was further supported by localization of the PfMDR1 protein to the DV membrane (Cowman 1991), but a number of complexities soon became apparent. Mapping of genes that determine CQ resistance in one cross had already shown that PfMDR1 could not be the primary determinant of CQ resistance in all parasite lines (Wellems 1991); moreover, *in vitro* selections for changes in CQ resistance yielded unexpected changes in *pfmdr1* copy number and revealed interesting changes in sensitivity to mefloquine, a distinct antimalarial drug that also acts in the DV (Barnes 1992). Subsequent studies that included DNA transfections and genetic crosses in both *P. chabaudi*, a mouse parasite, and *P. falciparum* continue to support a role of the *pfmdr1* product in resistance certainly to mefloquine, quinine, and halfantrine with lesser, but significant, effects on susceptibility to chloroquine and artemisinin (Reed 2000; Cravo 2003; Sa 2009).

Although both of these putative transporters have been actively studied for some time, two important questions have not been adequately addressed and are intensively debated to this date. First, how do mutations or altered expression of one or both of these proteins yield parasite resistance to chloroquine and other antimalarial drugs? This question has been difficult to resolve, partly because of the multitude of factors that can influence CQ accumulation in infected red cells. Four membranes separate the DV lumen from host plasma. Moreover, because CQ is a weak base with two ionization sites ($pK_a = 8.1$ and 10.2), aqueous solutions contain several distinct isoforms of CQ in amounts that vary with local pH; each isoform has a distinct net charge and membrane permeability. Therefore, studies that use tracer accumulation to quantify CQ transport are vexed by unknown distribution within compartments of infected cells, presumed trapping of charged forms in acidic compartments, and uncertain rates of free diffusion of the unprotonated form across membranes. An additional complexity is that, once in the DV, CQ binds ferriprotoporphyrin IX and possibly phospholipids; this binding also influences the transport kinetics and apparent steady-state accumulation.

In addition to heroic studies of CQ accumulation in infected erythrocytes, a number of groups have used heterologous expression of *pfcrt* or *pfmdr1* to examine drug transport free of these parasite-specific complexities (Volkman 1995; Zhang 2002; Nessler 2004; Naude 2005; Sanchez 2008). These studies largely support a model where the expressed protein functions as a transporter capable of directly mediating transmembrane movement of CQ and/or other drugs, but indirect mechanisms such as transport of acid equivalents followed by redistribution of antimalarial drugs have not been excluded. The precise mechanism of transport (channel vs. carrier), the energy dependence of transport (coupling to other solutes vs. driven by ATP hydrolysis), and other key properties are all still debated.

The second important question about these two transporters relates to their physiological role in the parasite. Neither has been successfully knocked out, suggesting that they are essential. They are presumed to function in the transport of one or more solutes at the DV membrane, but what the solutes are remains unclear. Hypotheses for PfCRT include a role in transport of amino acids or short peptides generated by hemoglobin digestion in the DV, H^+ transport as required for acidification of the DV, conductive cation or Cl^- movement, glutathione transport, or that the protein is a

nonspecific activator of transporters (Zhang 2004; Nessler 2004; Maughan 2010). There are also a number of proposed physiological roles for PfMDR1 (Rohrbach 2006). Unfortunately, heterologous expression of these transporters has not yet yielded definitive insights into either function or antimalarial resistance mechanism.

Nevertheless, the history of drugs that act upon the DV makes these transporters attractive targets for future therapeutics. Several groups have carried out medicinal chemistry based on the substituted quinoline scaffold. Effective derivatives that overcome the parasite's resistance mechanisms may circumvent presumed export of the antimalarial by PfCRT or PfMDR1, function as chemosensitizers to selectively restore drug accumulation in CQ-resistant parasites, or interact more avidly with DV targets (Kaur 2010; Peyton 2012). Some of these compounds are effective against multidrug-resistant parasites and have excellent potential to provide key insights into DV physiology. However, given that related compounds have already selected for resistance mutations in the field, an important concern with these new drug leads is that they might also succumb to acquired resistance. A better understanding of transport mechanisms and physiological roles served by PfCRT and PfMDR1 might be the most important step in rational drug discovery targeting this site.

Mitochondrial inner membrane: An unusual ATP synthase with uncertain function

Asexual *P. falciparum* parasites have a small single mitochondrion with few cristae and a reduced mitochondrial genome; moreover, biochemical measurements have suggested little or no oxidative phosphorylation (Fry and Beesley 1991), with nearly all the glucose consumed being converted to lactic acid through anaerobic glycolysis (Roth 1990). Nevertheless, the enzymes required for a complete TCA cycle are retained and appear to function in the production of biomolecules (Macrae 2013; Storm 2014). The enzymes for mitochondrial electron transport are also intact; they are the targets of the synergistic antimalarial drugs atovaquone and proguanil, as well as a number of other drugs in development (Painter 2007; Deng 2009; Rodrigues 2010).

A key function of electron transport in most eukaryotes is to create an inner mitochondrial membrane H^+ gradient that drives ATP synthesis on a remarkable transporter known as ATP synthase, which consists of two motors, F_1 and F_0. F_0 contains a membrane-embedded H^+ transporter that rotates like a mill wheel as protons move down their gradient from the intermembrane space into the mitochondrial matrix (Figure 11.1B). F_1 captures and stores this energy by synthesizing ATP from ADP and inorganic phosphate. Several components of this multi-subunit complex, including the essential a and b subunits of the F_0 motor, were not detected by bioinformatics surveys of the parasite genome, initially suggesting that malaria parasites had evolved to live without oxidative phosphorylation. However, oxidative phosphorylation has been detected in rodent malaria parasites (Uyemura 2004), in other apicomplexans (Vercesi 1998), and in *Tetrahymena thermophilia*, whose genome also lacks unambiguous candidates for some of the same subunits (Balabaskaran 2010). Also suggestive were DNA transfection experiments that could not disrupt either the β or γ subunits from *P. falciparum* cultures (Balabaskaran 2011).

These paradoxical findings were first reconciled through proteomic studies that identified several of the missing subunits in *Tetrahymena*; the novel subunits fulfill structural requirements for ATP synthesis and yield an altered ultrastructure of the complex in electron microscope studies (Balabaskaran 2010). Unfortunately, malaria parasites lack clear orthologues of the subunits identified in *Tetrahymena*. Subsequent biochemical studies by the same group used *P. falciparum* cultures to recover a high-molecular-weight F_1F_0-ATP synthase complex that forms dimers resembling those

of conventional ATP synthases (Balabaskaran 2011). DNA transfection studies confirmed localization of multiple subunits to the parasite mitochondrion and suggested that assembly of the complex is essential for *in vitro* parasite survival.

It remains unclear whether the malaria parasite ATP synthase can be targeted for development of antimalarial drugs. The key hurdle remains determining what, if any, role this complex serves for the parasite. Possibilities include ATP synthesis under stage- or environment-specific conditions, contribution to the mitochondrial membrane potential via ATP hydrolysis and H^+ extrusion, and/or a role in maintaining the structure of the mitochondrion. The unusual composition of this enzyme in alveolates, its refractoriness to genetic disruption, and the success of mitochondrial-specific drugs suggests that ATP synthase should not yet be dismissed as an antimalarial target.

Conclusions

Malaria parasites are highly adapted for growth and replication within host erythrocytes. This specialized niche requires a unique collection of transmembrane transport activities, some of which we have highlighted in this review. The unusual properties of these transporters, along with the critical roles they are thought to serve for the parasite, suggest that they may be ideal targets for antimalarial therapies. For each chosen transporter, we have reviewed the evidence for facilitated transmembrane transport, features that distinguish the parasite transporter from similar activities in other systems, reasons that the transporter may be a good drug target, and scientific uncertainties relevant to the drug-development process. Advances in parasite genomic, transfection, and biochemical technologies may be instrumental in translating these basic research findings into future antimalarial drugs.

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CHAPTER 12

The proteolytic repertoire of malaria parasites

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Proteases are enzymes that hydrolyze peptide bonds in substrate proteins. A large number of proteases with crucial functions have been identified in many infectious organisms, and proteases represent attractive drug targets. As proteases have well-defined active sites, small molecule inhibitors based on these active sites can be designed to block protease activities associated with disease conditions, including parasitic diseases. Several protease inhibitors are already in clinical use for treatment of human diseases, which supports drug-discovery efforts targeting proteases (Drag 2010).

Malaria parasite development follows a complex multistage life cycle that involves as many as 15 distinct stages. Early studies using protease inhibitors suggested critical roles for all five classes of proteases (aspartic, cysteine, metallo, serine, and threonine) in malaria parasite development at multiple stages, including regulated processing of parasite and host proteins. Genome-wide analysis of all *Plasmodium* species sequenced so far has revealed that the *Plasmodium* proteolytic repertoire contains 115 to 137 putative proteases of all five classes (Table 12.1) (Kuang 2009). However, only a handful of these proteases have been studied, and only a few of the biochemically characterized proteases have been assigned functions in parasite biology.

The following sections are about the proteases that have been characterized in malaria parasites; most of the characterized proteases belong to *P. falciparum*, and some of the proteases described below are also covered in several reviews (Rosenthal 2011; Ersmark 2006; Withers-Martinez 2004; Dowse 2005; Skinner-Adams 2010; Klemba 2002; Coombs 2001; Mc Kerrow 2008).

Aspartic proteases

Aspartic proteases are so named because aspartate residues in these proteases act as ligands and activate a water molecule that makes a nucleophilic attack on the scissile peptide bond in the substrate protein. The archetype aspartic protease is pepsin, which belongs to the family A1. It has two topologically similar lobes, with the active site in between the lobes. The active site is formed by a dyad of aspartate residues, with each lobe contributing one aspartate residue. A water molecule is bound to and activated by the aspartate dyad at the time of catalysis, which acts as a nucleophile. The predicted and characterized aspartic proteases of *P. falciparum* include the pepsin-like plasmepsins and a presenilin-like signal peptide peptidase (SPP).

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Table 12.1 Proteolytic repertoire of malaria parasites.

Catalytic type	Protease family	Representative *Plasmodium* protease	Protease genes in the genome		Role in *Plasmodium* biology
			Pf	Pb	
Aspartic	A1 (pepsin)	Plasmepsins	11	10	Hemoglobin hydrolysis and processing of exported proteins (Banerjee 2002; Russo 2010; Boddey 2010).
	A2 (HIV-1 retropepsin)	DNA damage inducible protein-1	1	1	Not known
	A22 (presenilin)	Signal peptide peptidase (SPP)	1	2	Roles in parasite growth and invasion (Li 2008, 2009; Marapana 2012)
Cysteine	C1 (papain)	Falcipains, DPAP, SERA	16	16	Hemoglobin hydrolysis, egress, processing of plasmepsins and gametocyte proteins (Rosenthal 2011; Klemba 2004; Arastu-Kapur 2008).
	C2 (calpain)	Calpain	1	1	Cell cycle progression (Russo 2009).
	C12 (UCH)	Not characterized	2	2	Not known
	C13 (legumain)	Not characterized	1	1	Not known
	C14 (caspase)	Metacaspase	3	4	Inhibition studies suggest a role in cell death (Le Chat 2007; Meslin 2011).
	C19 (UCH)	PfUCHL3	10	12	Seems to be essential for erythrocytic stage development (Artavanis-Tsakonas 2006, 2010).
	C48 (Ulp1endopeptidase)	Not characterized	2	2	Not known
	C50 (separase)	Not characterized	1	1	Not known
	C54 (Aut2 peptidase)	Not characterized	1	N/A	Not known
	C56 (PfpI endopeptidase)	PlasmoDJ1	1	1	Anti-oxidative function (Singhal 2014)
	C65 (otubain-1)	Not characterized	1	1	Not known
	C97 (PPPDE peptidase)	Not characterized	1	1	Not known
Metallo	M1 (aminopeptidase N)	M1-family alanyl aminopeptidase (PfA-M1)	2	1	Hemoglobin degradation in the food vacuole (Ragheb 2011; Harbutt 2011).
	M3 (thimet oligopeptidase)	Not characterized	2	2	Not known
	M14 (carboxypeptidase A)	Not characterized	1	1	Not known

Table 12.1 (Continued)

Catalytic type	Protease family	Representative *Plasmodium* protease	Protease genes in the genome		Role in *Plasmodium* biology
			Pf	Pb	
	M16 (pitrilysin)	Falcilysin	6	5	Degradation of hemoglobin peptides in the food vacuole, and processing of the transit peptide in the apicoplast (Eggleson 1999; Ponpuak 2007).
	M17 (leucyl aminopeptidase)	Pf-LAP	1	1	Degradation of hemoglobin peptides in the cytoplasm (Stack 2007; Gardiner 2006).
	M18 (aminopeptidase I)	Aspartyl aminopeptidase	1	2	Degradation of hemoglobin peptides and erythrocyte cytoskeleton proteins (Teuscher 2007; Lauterbach 2008).
	M22 (O-sialoglycoprotein peptidase)	Not characterized	2	3	Not known
	M24 (methionyl aminopeptidase 1)	PfMetAP1b and PfMetAP2	5	6	Inhibitors block erythrocytic stage development of *P. falciparum* (Chen 2006, 2009).
	M41 (FtsH endopeptidase)	Not characterized	3	3	Not known
	M50 (S2P protease)	Not characterized	3	2	Not known
	M67 (Poh1 peptidase)	Not characterized	3	3	Not known
Serine	S1 (chymotrypsin)	Not characterized	2	N/A	Not known
	S8 (subtilisin family)	PfSUB1, PfSUB2, and PfSUB3	3	5	PfSUB1has essential roles in egress and invasion of erythrocytes by merozoites (Yeoh 2007; Koussis 2009; Blackman 1993; Breton 1992). PfSUB2 processes MSP-1 and AMA1 (Harris 2005).
	S9 (prolyl oligopeptidase family)	Not characterized	1	1	Not known
	S14 (ClpP endopeptidase family)	PfClpP	5	2	Has crucial role in parasite development (Rathore 2010).

(*Continued*)

Table 12.1 (Continued)

Catalytic type	Protease family	Representative *Plasmodium* protease	Protease genes in the genome		Role in *Plasmodium* biology
			Pf	Pb	
	S16 (lon protease family)	Not characterized	1	4	Not known
	S26 (signal peptidase I family)	Signal peptidase	2	2	The *P. falciparum* type I signal peptidase has been shown to be active (Sharma 2005).
	S33 (prolyl aminopeptidase)	Not characterized	3	1	Not known
	S54 (Rhomboid family)		8	8	Mediate shedding of a number of surface proteins (Baker 2006; O'Donnell 2006; Ejigiri 2012; Lin 2013).
Threonine	T1 (proteasome family)	Proteasome with β1, β2, and β5 peptidases	3	3	Inhibition studies indicate crucial roles in multiple stages (Gantt 1998; Czesny 2009; Lindenthal 2005; Aminakea 2012).
		ClpQY	1	1	Crucial for mitochondrial homeostasis (Jain 2013).
Unknown	U48 (prenyl protease 2 family)		1	1	Not known

Plasmepsins

Plasmepsins are the best-studied aspartic proteases of malaria parasites, and there are 10 plasmepsins in *P. falciparum*. Seven plasmepsins (I, II, IV, V, IX, X, and a histoaspartic protease [HAP]) are predominantly expressed during erythrocytic stage development of *P. falciparum* (Coombs 2001; Mc Kerrow 2008). Plasmepsin I, II, and IV and HAP are present in the food vacuole, a lysosome-like organelle of the parasite, and plasmepsin V is a membrane-bound enzyme in the endoplasmic reticulum (McKerrow 2008). Biochemical properties and functions of plasmepsins VI through X are not known. All other malaria parasites sequenced to date lack plasmepsins I and II and HAP, and they thus contain only seven plasmepsins (Dame 2003).

The food-vacuole plasmepsins, plasmepsin V, and likely the rest of the plasmepsins, are produced as proenzymes, with an amino-terminus proregion and a carboxy-terminus protease region (Figure 12.1). The N-terminus of the proregion contains a transmembrane domain. Plasmepsin V also contains a hydrophobic domain in the carboxy-terminal of the protease region, which together with the transmembrane domain in the N-terminal proregion is likely responsible for localization of this protease in the endoplasmic reticulum as an integral membrane protein (Klemba 2005).

The protease regions, except that of HAP, contain two motifs containing the catalytic aspartate residues. HAP is unique in having a histidine residue instead of the aspartate residue (HTAS in place of DTGS) in the first motif, and both native and recombinant HAP have been shown to possess pepstatin A-inhibitory activity (Banerjee 2002; Xiao 2006).

All food vacuole plasmepsins have been crystallized with or without inhibitors, and they share a typical bilobed pepsin-like structure (Bhaumik 2012). The proenzyme forms of the food vacuole

Protease			Length (aa)
PMI	DTGS	DSGT	452
PMII	DTGS	DSGT	453
HAP	HTAS	DSAT	451
PMIV	DTGS	DSGT	449
PMV	DTGS	DSGS	590
PMVI	DTGS	DTGS	432
PMVII	DTGS	DTGT	450
PMVIII	DTGS	DTGT	385
PMIX	DTGS	DSGT	627
PMX	DTGS	DTGT	573

Figure 12.1 Domain organization of plasmepsins. Shown on the top is the general architecture of *P. falciparum* plasmepsins, with prodomain (Pro) and mature protease domain (Protease). The hydrophobic transmembrane region (TM) in the prodomain and active site motif 1 and motif 2 (star) in the protease domain are indicated. The names of plasmepsins sharing this architecture, the sizes, and the sequences of active site motifs are shown below the schematic. The sizes are scaled (≈150 amino acid residues/inch) and the amino acid numbers are based on the protease sequences on the PlasmoDB.

plasmepsins undergo processing upon reaching the food vacuole, releasing the mature proteases. Falcipain cysteine proteases (see below) have been suggested to be the key mediators of processing of the plasmepsin zymogens by cleaving within a conserved motif ((Y/H)LG↓(S/N)XXD) (Francis 1997; Drew 2008; Banerjee 2003). However, all four plasmepsins undergo processing upon treatment of parasites with cysteine protease inhibitors, suggesting that autoprocessing is an alternative pathway, which could be used under certain conditions and/or as an accessory mechanism contributing to processing.

Plasmepsins I, II, HAP, and IV have been shown to degrade haemoglobin *in vitro* under acidic conditions, a feature consistent with their maximum activity in the acidic pH range (4.5–6) and localization in the food vacuole, which suggested their role in hemoglobin hydrolysis (Banerjee 2002; Goldberg 1991; Gluzman 1994; Dame 1994). However, gene knockout studies show that the food vacuole plasmepsins are dispensable for the erythrocytic development of *P. falciparum*, and the knockout parasites apparently had normal hemoglobin degradation, indicating accessory nonessential role of the food vacuole plasmepsins in hemoglobin catabolism (Bonilla 2007; Bonilla 2007; Liu 2005). Notably, parasites lacking all the food vacuole plasmepsins had a slight growth disadvantage as compared to the wild-type parasites and accumulated multi-lamellar bodies in the food vacuole, suggesting a role of the food vacuole plasmepsins in digestion of vesicles inside the food vacuole (Bonilla 2007). In addition to asexual intraerythrocytic stages, plasmepsin IV has also been shown to be expressed in sexual and ookinete stages of *P. gallinaceum*, and a role for it has been suggested in oocyst development (Li 2010).

Plasmepsin V is present in the endoplasmic reticulum, where it processes a set of proteins that are exported outside the parasite (Klemba 2005; Russo 2010; Boddey 2010). A large number of exported proteins contain a conserved five–amino acid motif (RxLxQ/E/D) known as the *Plasmodium* export element (Pexel) or host targeting (HT) signal (Hiller 2004; Marti 2004). The Pexel/HT motif is essential for transport of exported proteins, and plasmepsin V specifically cleaves within the Pexel/HT motif, which has been shown to be a prerequisite for the transport (Chang 2008; Boddey 2010; Russo 2010). Plasmepsin V cleaves the RxL↓xQ/E/D motif with restricted cleavage specificity, because replacement of the P3 R or P1 L in the motif, even with homologous residues K and I, respectively, makes the motif resistant to cleavage. Plasmepsin V is present in all studied species of malaria parasites, appears to be essential for parasite development, does not have a closely related homologue in the host, and follows strict substrate specificity, which together make it an attractive drug target.

Presenilin-like protease

The presenilin-like signal peptide peptidase of *P. falciparum* (PfSPP) is expressed during erythrocytic-stage infection, and it is suggested to be a micronemal protein (Li 2008, 2009). Decreased invasion of erythrocytes by merozoites in the presence of anti-PfSPP antibodies and a block in invasion and parasite development by inhibitors of the mammalian homologue of PfSPP suggested a key role for it in invasion and parasite development (Li 2008, 2009). However, a subsequent study showed that PfSPP resides in the endoplasmic reticulum, and it appears to be required for parasite growth but not for invasion (Marapana 2012). Nonetheless, PfSPP is a promising drug target.

Cysteine proteases

The largest group of proteases in *Plasmodium* is the cysteine proteases, which are so named because they use a cysteine residue to hydrolyze the scissile peptide bond in substrates. The active site contains the catalytic cysteine residue and a histidine residue, and it may also contain additional residues depending on the protease type. The best-known cysteine protease is the plant protease papain, and its best-studied homologues in *Plasmodium* are hemoglobinases, which are known as falcipains in *P. falciparum*. The genomes of malaria parasites contain multiple cysteine protease genes. *P. falciparum* contains 27 putative cysteine proteases, including four falcipains, three dipeptidyl aminopeptidases (DPAP), and nine serine-repeat antigens (SERA). The best-studied proteases include falcipains and their homologues in other malaria parasites.

Falcipains

Falcipains are the best-studied cysteine proteases of *P. falciparum* and include falcipain-1 (FP1), falcipain-2A (FP2A), falcipain-2B (FP2B), and falcipain-3 (FP3). The sequence identity between FP1 and the other three falcipains is 28% to 30%; FP2A and FP2B are nearly identical (92%) and share 54% identity with FP3. There are four homologous proteases in *P. vivax*, called vivapains (VP1–4); four in *P. knowlesi*, called knowpains (KP1–4); and two in the rodent malaria parasite *P. berghei*, called berghepains (BP1 and BP2) (Singh 2002; Na 2004, 2010). FP1 orthologues are present in single copies and are clearly identifiable; the remaining three falcipains and their homologues are all quite similar in sequence and will be called FP2/3 orthologues henceforth.

All falcipains and their *Plasmodium* homologues share the typical zymogen-like prodomain-mature protease domain organization of papain-like proteases, with a hydrophobic region in the beginning of the prodomain and conserved catalytic amino acid residues (Q, C, N, H, and W) in the mature protease domain (Figure 12.2). The prodomains of falcipains are longer than those of most papain-like proteases. They contain ERFNIN and GNFD motifs, which are present in the majority of cathepsin L–like proteases and have been shown to inhibit their cognate proteases in cathepsin L–like proteases and FP2 (Karrer 1993; Coloumbe 1996; Pandey 2009). The FP2/3 subfamily contains unique features in the mature domain, which include a refolding domain in the beginning of the mature protease domain and a hemoglobin-binding insert in the C-terminus between highly conserved catalytic histidine and asparagine residues (Sijwali 2002; Pandey 2004; Pandey 2005; Wang 2006).

All four falcipains are expressed during erythrocytic-stage parasite development and are located in the food vacuole, a lysosome-like organelle wherein hemoglobin is degraded (Shenai 2000; Sijwali 2001; Rosenthal 2009; Singh 2006; Salas 1995; Jeong 2006). Recombinant falcipains are maximally active around the food vacuole pH (pH 5.5), degrade hemoglobin, and are inhibited by cysteine protease inhibitors that also block parasite development, which indicates their role in hemoglobin degradation. FP2/3 subfamily proteases of other malaria parasites have also been characterized,

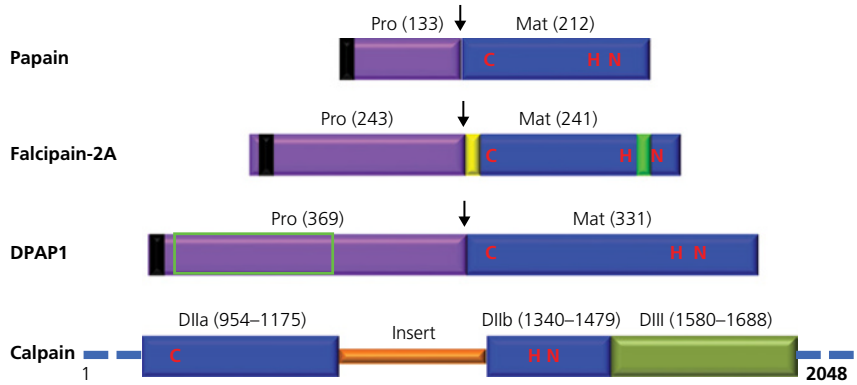

Figure 12.2 Domain organization of cysteine proteases. Shown is a comparison of general architectures of different *P. falciparum* cysteine proteases with papain. The prodomains (Pro) and mature protease domain (Mat) are indicated with hydrophobic transmembrane regions (black bars), the active site amino acid residues (C, H, and N), processing site (arrow), and the number of amino acid residues or boundary of the indicated region (bracket). Falcipain-2A is a representative of the FP2/3 subfamily proteases of malaria parasites, which differ from all other known papain-like proteases in having the refolding (yellow) and hemoglobin-binding domains (green). DPAP1 and other DPAPs, as in human cathepsin C, contain a putative exclusion domain (green box) that confers exopeptidase dipeptidyl activity to cathepsin C-like proteases. The *Plasmodium* calpain is a giant protease with long amino-terminal and C-terminal extensions (broken bar), the protease region (DIIa and DIIb) is interrupted by an insert, and the Ca^{2+}-binding region (DIII) is indicated. The active site residues (C, H, and N) common to all papain-family proteases are indicated. The sizes are scaled (≈150 amino acid residues/inch) and the amino acid numbers are based on the protease sequences on the PlasmoDB.

which showed their biochemical and subcellular localization similarity with FP2 and FP3 (Singh 2002; Na 2004, 2010; Salas 1995; Prasad 2012). The FP2/3 subfamily proteases are also implicated in egress of merozoites from infected erythrocytes (described in Chapter 7). Unlike the bulk degradation of hemoglobin, the FP2/3 subfamily proteases are also implicated in selective processing of several parasite proteins, including plasmepsins and the gametocyte surface protein Pfs230. Furthermore, the inhibition of non–erythrocytic-stage parasite development by cysteine protease inhibitors suggests additional roles of FP2/3 subfamily proteases (Brooks 2000; Eksi 2007; Rupp 2008; Copi 2005).

Despite substantial progress in biochemical understanding of FP2/3 subfamily proteases, it is not clear if they have distinct roles in parasite development. Gene-knockout studies demonstrated that of the four falcipains, FP1 and FP2B are dispensable, FP2A is the key hemoglobin-degrading protease, and FP3 is essential for erythrocytic-stage development of *P. falciparum* (Sijwali 2004; Sijwali 2004, 2006). Independent studies, in which the FP2A gene was knocked out or its expression level was reduced, support a crucial role of FP2A in hemoglobin degradation (Malhotra 2006; Liu 2006; Armstrong 2007). Taken together, inhibition of parasite development by cysteine protease inhibitors and indispensability of FP3 strongly support drug target potential of falcipains.

Dipeptidyl aminopeptidases

Dipeptidyl aminopeptidases (DPAPs) are homologues of human cathepsin C, which sequentially cleaves dipeptides from the N-terminus of oligopeptides. *P. falciparum* encodes three dipeptidyl aminopeptidases (DPAP1–3); DPAP1 and DPAP3 have been characterized and shown to be expressed during erythrocytic stage development (Klemba 2004; Arastu-Kapur 2008). PfDPAP1 is present in the food vacuole, degrades dipeptides efficiently at acidic pH, and appears to be essential for parasite development, which implicates it in degradation of oligopeptides generated upon digestion of

hemoglobin by falcipains and plasmepsins. PfDPAP3 is expressed in later stages, and its specific inhibition by a small molecule blocked egress from the erythrocyte, suggesting a role for it in this process. Thus, essentiality of DPAP1 and DPAP3 supports their potential as antimalarial drug targets.

Calpain

Calpains are Ca^{2+}-dependent cysteine proteases that contain two domains: the papain-like protease domain with conserved active site residues (C, H, N) and the Ca^{2+}-binding regulatory domain. Based on the concentration of Ca^{2+} required for activation, there are two major types of calpains: μ-calpain and m-calpain, which require micromolar and millimolar concentrations of Ca^{2+}, respectively. All malaria parasites have a single calpain of the predicted size of 242 kDa. The *P. falciparum* calpain (Pfcalpain) is expressed in early stages of erythrocytic development. It could not be knocked out, but conditional knockdown greatly impaired progress beyond the ring stage, which suggested its role in cell cycle progression (Russo 2009). As the parasite calpain seems to be a distant homologue of host calpains and because it is essential for parasite development, it could be a target for development of antimalarials.

Serine repeat antigen (SERA)

SERA proteins belong to a multigene family, which shares a papain-like protease domain, with partial conservation of active-site residues. The first characterized member of this family was identified as an antigen present in parasites undergoing rupture/invasion, and it was termed p126 (Delplace 1985; Aoki 2002). Cloning of the p126-encoding gene revealed that the encoded protein has repeats of serine, and hence it was termed serine repeat antigen (SERA) or serine stretch protein (SERP) (Bzik 1988; Knap 1989). The second member was identified a little later; it was found to be highly similar to SERP, but did not have the serine repeat, and it was called highly homologous to the serine stretch protein (SERPH) (Knapp 1991).

The availability of genome sequences of malaria parasites and independent cloning studies have revealed the presence of multiple SERA proteins in all malaria parasites (Bzik 1988; Knapp 1991; Gardner 1998; Gor 1998; Kiefer 1996; Arisue 2007), with nine members in *P. falciparum* and five members in *P. berghei*. Of the nine SERAs, eight members are arranged in tandem on chromosome 2 (SERA1-8), and SERA9 is present on chromosome 9 (Miller 2002); the originally identified SERA and SERPH are now called SERA5 and SERA6, respectively. Only SERA5 has the serine stretch, but all members share the papain-like protease domain and are most similar in this region (Figure 12.2). Importantly, the active site residues of papain-like proteases (Q, C, H, N in papain and falcipains) are positionally conserved in SERA6–8, but the remaining SERA proteins have serine in place of the catalytic cysteine residue (Q, S, H, N). Similarly, of the five SERAs in *P. berghei*, the catalytic cysteine residue is retained in three (PbSERA3-5) and replaced with serine in two (PbSERA1 and PbSERA2) proteins.

Expression and localization studies indicated that SERA3-6 and SERA9 are expressed during erythrocytic stage development (Aoki 2002; Miller 2002), and SERA3–6 are present in the parasitophorous vacuole (Aoki 2002; Knapp 1991; Delplace 1988).

A large number of studies have demonstrated the presence of SERA-reactive antibodies in humans living in malaria-endemic regions, which supports the vaccine potential of SERAs, particularly SERA5 and SERA6 (Aoki 2002; Knapp 1991; Miller 2002; Delplace 1988).

Knockout studies of SERA genes have demonstrated that SERA2, 3, 7, and 8 are dispensable and that SERA4, 5, and 6 are essential for erythrocytic stage development of *P. falciparum* (Miller 2002). Individual knockouts of SERA1 and 2 in *P. berghei* and *P. yoelii* were successful (Putrianti 2010; Huang 2013). However, knockout of PbSERA5 (also known as the egress cysteine protease 1 [ECP1]), an orthologue of PfSERA8, resulted in complete block of sporozoite egress from oocysts (Aly 2005).

SERA6–8 and their homologues are expected to be proteases, because these proteins have all the positionally conserved active-site residues of papain-family proteases (C, H, N). Although a direct

demonstration of protease activity for SERA6–8 remains to be done. PbSERA3, the orthologue of PfSERA6, has been shown to undergo processing upon activation by SUB1 (Ruecker 2012). SERA5 has been shown to undergo processing, and the bacterially expressed protease domain of SERA5 showed marginal chymotryptic activity against peptide substrates, suggesting that SERA5 has protease activity (Hodder 2012). The presence of serine in place of the catalytic cysteine supports the chymotryptic activity of SERA5.

Early studies on SERA5 showed formation of merozoite and schizont clusters in the presence of SERA5 antibodies, causing a block in merozoite release, which suggested a role of SERA5 in egress (Delplace 1988). Two studies indicated that PfSUB1 most likely processes all SERA proteins and also showed that inhibition of the PfSUB1-mediated processing of SERA5 blocks egress, which together support a role for SERA 5 and perhaps other SERAs in egress (Yeohh 2007; Koussis 2009).

Considering the multiple lines of evidence described above and cited in the references herein, SERA proteins appear to have essential roles in parasite development at multiple stages, and they represent unique targets for vaccine and drug development.

Metalloproteases

Peptidases that use a metal ion–activated water molecule for cleaving scissile peptide bonds in substrates are known as metalloproteases. A divalent metal cation (usually Zn^{2+}, or Cu^{2+}, Ni^{2+}, Mn^{2+} in some metalloproteases) is usually bound to three amino acid residues (His, Glu, Asp, or Lys) of the protease. Malaria parasite genomes encode for several putative metalloproteases, with 29 predicted to be present in *P. falciparum*, and a few of these have been characterized, as described below.

Alanyl aminopeptidase

All malaria parasites contain an alanyl aminopeptidase, which in *P. falciparum* is called M1-family alanyl aminopeptidase (PfA-M1). It was first identified as an M1 family zinc-metallopeptidase and was shown to be present in the cytoplasm of erythrocytic–stage parasites (Florrent 1998). A subsequent study showed that it is present both in the parasite cytoplasm and in the food vacuole and has an aminopeptidase activity with broad substrate specificity (Allary 2002). More-recent studies showed that PfA-M1 is present in the food vacuole and nucleus, but not in the parasite cytoplasm (Dalal 2007; Ragheb 2011) and suggested that the cytoplasmic localization observed in previous studies (Florrent 1998; Allary 2002) could be due to fixation conditions that did not preserve cellular organelles.

PfA-M1 is active in a broad pH range, including the food vacuole pH (5.0–5.5), cleaves a variety of amino acid residues from the N-terminus of peptides, and is inhibited by the metalloprotease inhibitor bestatin, suggesting that it degrades hemoglobin peptides in the food vacuole (Allary 2002; Ragheb 2011; Poreba 2012). Inhibition of PfA-M1 in parasites by a specific inhibitor, (Benzyl)Tyr-Ala-ABP (BTA), supported its role in degradation of hemoglobin-derived peptides in the food vacuole, as the inhibited parasites had several-fold more hemoglobin-derived peptides than untreated parasites (Harbutt 2011).

PfA-M1 is 1085 amino acids long, with an N-terminus of 194 amino acid residues, and the remaining C-terminal region contains the protease domain (Figure 12.3). The N-terminus contains a hydrophobic domain that likely has a key role in targeting the protein. The native protein is around 120 kDa, which appears to be processed into 68-kDa and 35-kDa forms. The 120-kDa and the 68/35-kDa forms have been suggested to be nuclear- and food vacuole–localized forms, respectively (Ragheb 2011). However, the function of PfA-M1 in the nucleus remains unknown.

The crystal structure of the C-terminal protease region revealed conserved $H^{496}EYFHX_{17}KE^{519}$ active site and $G^{490}AMEN$ substrate-binding motifs (McGowan 2009). The catalytic zinc ion is held in the active site by His^{496}, His^{500}, and Glu^{519} residues. Both inhibition and genetic studies support an

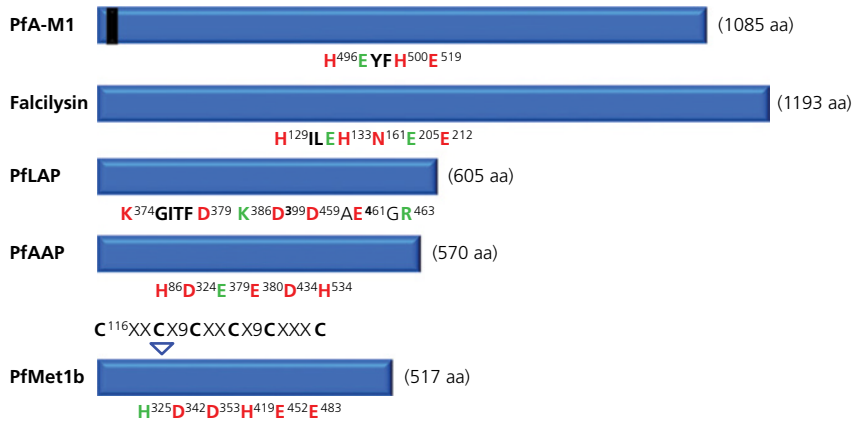

Figure 12.3 Schematics of the *P. falciparum* metallopeptidases. The indicated metallopeptidases are shown with the total number of amino acid residues in brackets. Active site residues are shown below the indicated protease, residues in red are putative metal binding ligands, and residues in green font are catalytic residues. PfA-M1 has a hydrophobic transmembrane region in the N-terminus (black bar), and PfMet1b has a Zn-finger motif (blue triangle). The sizes are scaled (≈150 amino acid residues/inch) and the amino acid numbers are based on the protease sequences on the PlasmoDB.

essential role of PfA-M1 in erythrocytic stage development, primarily in degradation of hemoglobin-derived peptides in the food vacuole, and thus indicate that it is an attractive drug target.

Falcilysin

Falcilysin is a pitrilysin-like protease belonging to the M16-family of metalloproteases, which are characterized by the presence of an HxxEH active site motif that binds with two Zn^{2+} ions (Figure 12.3). Falcilysin was first purified from the food vacuole fraction of *P. falciparum* erythrocytic stage parasites; the native enzyme was optimally active at pH 5.2 and degraded peptides derived from hemoglobin, which suggested a role for it in hemoglobin degradation in the food vacuole (Eggleson 1999). Falcilysin is 1193 amino acids long and about 130 kDa in size, and it is localized both in the food vacuole and the apicoplast, a chloroplast-like organelle in malaria parasites. It degraded peptide substrates both under acidic and neutral pH conditions, with remarkably different substrate specificity (Murata 2003a; Murata 2003b; Ponpauk 2007). Based on its localization and efficient degradation of hemoglobin-derived peptides at acidic pH and the transit peptide of an apicoplast-targeted protein at neutral pH, falcilysin has been suggested to be a dual-specificity protease that participates in hemoglobin catabolism in the food vacuole and degrades transit peptides in the apicoplast (Ralph 2007). The falcilysin gene could not be knocked out, but it was readily replaced by a functional allele, which together suggest its essentiality for parasite development and potential as a drug target (Ponpauk 2007).

Leucyl aminopeptidase

A leucyl aminopeptidase is conserved in all malaria parasites, and the *P. falciparum* homologue (PfLAP) has been shown to be expressed in all erythrocytic stages as a cytoplasmic protein (Dalal 2007; Stack 2007). PfLAP is 605 amino acids long (Figure 12.3); recombinant PfLAP (corresponding to amino acid residues 83–598) is active in the neutral-to-alkaline pH range, and it cleaves bulky hydrophobic amino acids, including leucine, from the N-terminus of peptides (Poreba 2012; Stack 2007).

Recombinant PfLAP exists as a hexamer, and each unit of the molecule has been proposed to contain two metal ions (most likely Zn^{2+}) bound to the active site motifs VGK^{374}GITFD^{379}SGG, MKFD^{399}MSG, and NTD^{459}AE^{461}GRL (Figure 12.3). The modeled structure of PfLAP based on the

crystal structure of the bovine lens leucine aminopeptidase (blLAP) predicted $D^{379}/D^{459}/E^{461}$ as ligands for the first metal ion and $K^{374}/D^{379}/D^{399}/E^{461}$ for the second metal ion (Stack 2007). In addition to these metal ligand residues, two residues (K^{386} and R^{463}) are predicted to be important for catalysis.

A leucyl aminopeptidase, most likely PfLAP, has been suggested to be one of the aminopeptidases that degrade hemoglobin-derived peptides in the cytoplasm to release free amino acids (Gavigan 2001). A recent study using a PfLAP-specific inhibitor, Phe-Naphthyl activity probe (PNAP), showed that inhibition of PfLAP kills parasites before hemoglobin degradation begins, and hence suggested that PfLAP has an essential role in addition to hemoglobin catabolism (Harbutt 2002). The PfLAP gene could not be disrupted, and a PfLAP-specific inhibitor blocked parasite development, which together make it an attractive drug target (Dalal 2007; Harbutt 2002; Gardiner 2006).

Aspartyl aminopeptidase

A cytosolic aminopeptidase with restricted specificity for cleavage of the N-terminus acidic amino acid residue (Asp and Glu) of proteins at neutral pH has been proposed to participate in degradation of hemoglobin peptides in the cytosol, and it is known as PfAAP for the *P. falciparum* aspartyl aminopeptidase (Teuscher 2007). PfAAP contains 570 amino acid residues, and both recombinant and native proteins exist as octamers wherein each monomer has active site motifs for two metal ions. PfAAP shows maximum sequence similarity with the M18-family of metallopeptidases, including conservation of the active site signature HDEDH with Glu^{379} as the putative catalytic residues ($H^{86}D^{324}E^{379}E^{380}D^{434}H^{534}$) (Figure 12.3). Knockdown of PfAAP using an antisense approach has been shown to inhibit parasite development, but disruption of the PfAAP gene did not have any effect on parasite development, indicating that PfAAP is dispensable for erythrocytic stage parasite growth (Dalal 2007; Teuscher 2007).

Methionyl aminopeptidase

Methionyl aminopeptidases are exopeptidases that cleave the initiator methionine residue in proteins. *P. falciparum* has four methionyl aminopeptidases: PfMetAP1a, PfMetAP1b, PfMetAP1c, and PfMetAP2. PfMetAP1a, PfMetAP1b, and PfMetAP1c are mitochondrial/bacterial type 1 methionyl aminopeptidases, which differ from PfMetAP2, a type 2 methionyl aminopeptidase, in that the latter contains a 64 amino acids–long insert in the C-terminal protease region (Chen 2006, 2009; Zhang 2002). As in several structurally characterized MetAPs, both the proteases contain a catalytic His residue and five highly conserved residues (two Asp, one His, and two Glu) that coordinate two metal ions to form the binuclear active sites (Figure 12.3).

Recombinant PfMetAP1b has been shown to be active, and a potent and selective inhibitor of recombinant PfMetAP1b also blocked erythrocytic development of *P. falciparum* and significantly decreased parasitemia in a mouse model, which provided the proof of concept for the drug-target potential of PfMet1b (Chen 2006). Recombinant PfMetAP2 did not show activity, but it bound to fumagillin and fumarranol, which inhibit human MetAP2. The two compounds also inhibited erythrocytic-stage development of *P. falciparum*, and fumarranol cured mice infected with *P. yoelii* (Chen 2009). Importantly, fumarranol did not inhibit activity of the three type 1 recombinant proteases, including PfMetAP1b, indicating that fumarranol inhibited parasite development by blocking PfMetAP2 activity, and hence PfMetAP2 seems to be a validated drug target.

Serine proteases

A serine residue in serine proteases acts as a catalytic amino acid for making nucleophilic attack on the scissile peptide bond. In addition to the catalytic serine, the active site contains two histidines or one histidine and one aspartate. Trypsin is a commonly used and archetype serine protease that

contains a catalytic triad of histidine, aspartate, and serine residues. Malaria parasite genomes encode several types of serine proteases, including the most-studied subtilisins, the intramembrane rhomboid proteases, and the apicoplast protease ClpP. Chemical inhibition and genetic studies indicate crucial roles of subtilisins and rhomboid proteases in erythrocytic stages, particularly in invasion of erythrocytes by merozoites (Blackman 2008; Carruthers 2005).

Subtilisins

The prototypes for the subtilisin family are serine proteases secreted into the medium by the *Bacillus* species to obtain nutrients. These proteases use a catalytic triad of Asp, His, and Ser residues, and they bind to Ca^{2+}, which stabilizes the structure and enhances activity. Inhibition studies long ago suggested a crucial role for serine proteases in erythrocytic-stage parasite development, particularly in invasion (Braun-Breton 1988; Hadley 1983; Dejkriengkhul 1983; Banyal 1981; Dluzewski 1986; Blackman 1992). *P. falciparum* has three subtilisins: PfSUB1, PfSUB2, and PfSUB3 (Figure 12.4). PfSUB1 has been extensively studied, and it was first identified in 1998 as an erythrocytic-stage protein present in the secretory organelles of merozoites (Blackman 1998); PfSUB2 was identified in 1999, and it was also shown to be present in the secretory organelles of merozoites (Barale 1999); PfSUB3 has also been shown to be expressed during erythrocytic-stage development, and recombinant PfSUB3 has been shown to be active (Alam 2012; Alam 2013).

PfSUB1 is 690 amino acids long, and it is produced as a zymogen in mature schizonts and merozoites (Blackman 1993). The N-terminal 329 amino acids of the zymogen form the preprodomain, with an upstream signal sequence; the remaining 361 amino acids of the C-terminus form the protease domain, with an active site containing the catalytic triad (Asp^{372}, His^{428}, and Ser^{606}) and the oxyanion hole (Asn^{520}) residues (Figure 12.4).

Native PfSUB1 has been shown to be rapidly processed to a 54-kDa species and then to a 47-kDa species that is present in dense granules. Recombinant PfSUB1 also showed a similar processing pattern *in vitro*. For both recombinant and native PfSUB1, the 54-kDa fragment remains noncovalently associated with a 31-kDa species that contains the prodomain and is a potent inhibitor of the cognate catalytic domain (Sajid 2000; Jean 2003). While the conversion to the 54-kDa fragment

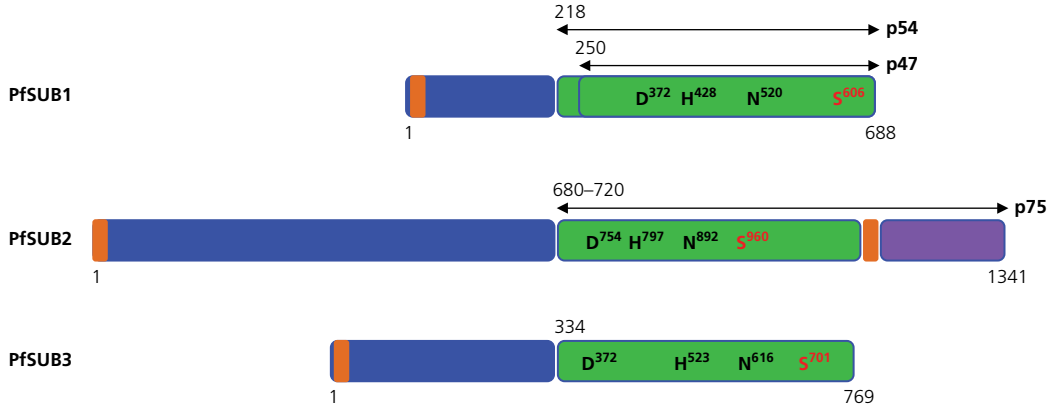

Figure 12.4 Schematic representation of the subtilisin domain organization. Shown are the zymogen forms with hydrophobic domains (orange), prodomains (blue), protease domains (green), cytoplasmic tail (lavender), and the residues forming the active site. The PfSUB1 zymogen is processed into the protease domain (p54) and then to the p47 form. The PfSUB2 zymogen is processed to the p75 form. The prodomain and protease domain boundaries of PfSUB3 have been predicted based on sequence analysis on the pfam database. The sizes are scaled (≈150 amino acid residues/inch) and the amino acid numbers are based on the subtilisin sequences on the PlasmoDB.

likely occurs in the endoplasmic reticulum, subsequent conversion to the 47-kDa species occurs upon/during transport to the Golgi, and this final form is present in dense granules at the apical complex of merozoites (Blackman 1993; Sajid 2000; Withers-Martinez 2002).

Recombinant PfSUB1 is maximally active at pH 8.1, and it prefers polar amino acid residues at the P1 position in substrates (Withers-Martinez 2002). The SUB1 protease of *P. vivax* has been characterized, and it showed a processing pattern and localization at the apical end of merozoites similar to PfSUB1 (Bouillon 2013). A study showed that PfSUB1 is not present in dense granules; rather it is discharged into the parasitophorous vacuole from novel organelles, called exonemes (Yeohh 2007). PfSUB1 has been proposed to be a multifunctional protease, with crucial roles in egress and invasion of merozoites (Reucker 2012; Yoeh 2007; Koussis 2009). It has been shown to process the SERA family of proteins in the parasitophorous vacuole to facilitate egress. PfSUB1 has also been shown to be involved in maturation of the proteins MSP1, MSP6, and MSP7, which is essential for invasion of erythrocytes.

PfSUB2 is 1337 amino acids long (Figure 12.4). It is predominantly expressed in schizonts and merozoites, and it is localized in micronemes of merozoites (Barale 1999). The native protein is about 150-kDa, which is processed to a 75-kDa (p75) intermediate form and then to a 70-kDa (p70) terminal form that persists in merozoites and most likely represents the active protease form (Hackett 1999). The p75 form has been predicted to be produced by cleavage between residues Tyr^{680}–Lys^{720}, but the identity of the N-terminus of the p70 form is yet to be established (Harris 2005).

PfSUB2 seems to be an integral membrane protein with a signal sequence at the N-terminus, a typical subtilisin active site containing catalytic triad (Asp^{754}, His^{797}, and Ser^{960}) and oxyanion hole (Asn^{892}) residues, and a transmembrane domain near the C-terminus (Figure 12.4). PfSUB2 has been suggested to process the MSP1 42-kDa fragment, leaving the membrane-bound MSP1 19-kDa fragment on the merozoite surface (Barale 1999).

The propeptide domains of subtilisins are potent inhibitors of their cognate mature proteases, and PfSUB2 is no exception; recombinant PfSUB2 propeptide has been shown to be a potent inhibitor of mature PfSUB2, and it also selectively reduced shedding of MSP1 and AMA1 (Harris 2005). The reduced shedding of AMA1 and MSP1 in the presence of PfSUB2 propeptide was most likely due to inhibition of AMA1 and MSP1 processing by PfSUB2, suggesting that these two proteins are processed by PfSUB2. Unsuccessful knockout of the *P. berghei* SUB2 gene suggests an essential function of SUB2 in erythrocytic parasite biology and supports its drug target potential (Uzureau 2004).

Rhomboid proteases

Rhomboid proteases are intramembrane proteases that make specific cuts in the membrane-spanning domains of substrate proteins. Although widely represented in prokaryotes and eukaryotes, these proteases share very low sequence similarity. The characteristic features of rhomboid proteases include the presence of six (mostly in bacteria) or seven (mainly in eukaryotes) transmembrane domains (TMDs), a catalytic dyad of Ser and His residues that are contributed by two separate TMDs, a WR motif in the first extracellular loop, and a HxxxxHxxxN motif (Figure 12.5). *Plasmodium* genomes encode eight rhomboid proteases, which are named ROM1, ROM3, ROM4, and ROM6–10 (Santos 2011; Dowse 2005; Baker 2006). Except for ROM8 and ROM10, all these proteases contain the characteristic GxSx and HxxGxxxG motifs in different TMDs. The predicted catalytic Ser-containing motif of ROM8 has Glu (ExSx) in place of Gly, and ROM10 contains the canonical catalytic HxxGxxxG motif. The number of putative TMDs is variable (Figure 12.5). ROM6 is predicted to be a mitochondrial protease; ROM1, 4, 7, and 8 are highly expressed in merozoites; PfROM3 is highly expressed in gametocytes (Baker 2006).

A number of proteins of the *Plasmodium* invasive stages that mediate adhesion to host cells, the adhesins, have been reported to be shed as a result of cleavage within or near their transmembrane domains; the molecular identity of the responsible shedases has begun to become clear. Some key adhesins are the apical merozoite antigen 1 (AMA1), the erythrocyte binding–like (EBL) family

Protease	TM	GxSx	HxxGxxxG	Length
ROM1	7	GAST	HLGGLISG	278 aa
ROM3	8	GAST	HIGGFLCG	267 aa
ROM4	7	GSSG	HIGGCLGG	759 aa
ROM6	7	GASG	HMFGLLGG	569 aa
ROM7	6	GSSS	HIVGFLCG	340 aa
ROM8	6	ESSS	HFFGFLGG	738 aa
ROM9	6	GASG	HLTGMGLG	488 aa
ROM10	6	GISP	?	274 aa

Figure 12.5 Schematic of the domain organization of *P. falciparum* rhomboids. The schematic of PfROM1 shows 7 transmembrane domains (orange rectangles), with the active site serine (S) and histidine (H) residues. The table shows key features (TM, number of transmembrane domains; the catalytic serine (GxSx) and histidine (HxxGxxxG) residue motifs; and the length of the protein) of the indicated rhomboids. The catalytic residues are in red type. Note that ROM8 has Glu in place of Gly in the GxSx motif, and ROM10 does not seem to have the catalytic HxxGxxxG motif, suggesting that these two are not active proteases. The sequences were analyzed for the presence of motifs on the MEROPS and Pfam databases, and transmembrane domains were predicted using the TMPred and TMHMM on the SACS (http://www.sacs.ucsf.edu/). The sizes are scaled (≈150 amino acid residues/inch) and the amino acid numbers are based on the protease sequences on the PlasmoDB.

proteins (EBA-175, BAEBL/EBA-140, MAEBL, and JESEBL/EBA-181), the reticulocyte binding–like (RBL) family proteins (Rh1, Rh2a, Rh2b, and Rh4), and the thrombospondin-related adhesive protein (TRAP) family proteins (TRAP, MTRAP, CTRP).

Coexpression of *P. falciparum* adhesins and selected rhomboid proteases in a heterologous eukaryotic expression system has revealed that AMA1 is cleaved by ROM1 only, and ROM1 and ROM4 can cleave a number of adhesins (Baker 2006). EBA-175 shedding, as a result of cleavage within a site that is conserved in all EBL family proteins, has also been shown to be mediated by ROM4 in *P. falciparum* (O'Donnell 2006). However, despite essentiality of AMA1 shedding for erythrocyte invasion, the ROM1 homologues in the rodent malaria parasites *P. berghei* and *P. yoelii* could be knocked out, although the knockout parasites had attenuated virulence, which suggests redundancy in ROM1 function (Vera 2011; Srinivasan 2009). ROM4 also cleaved the TMD of the major sporozoite invasin TRAP *in vitro,* and mutations within the cleavage site abrogated the shedding, resulting in a dramatic decrease in gliding motility of sporozoites, indicating that shedding of TRAP is crucial for motility (Baker 2006; Ejidiri 2012). Furthermore, the TRAP-shedding defective mutant also showed significant loss of infectivity when injected into the host intradermally, which is in agreement with the previously reported key role of TRAP in gliding motility and in vivo infectivity.

In a systematic gene-knockout study of the *P. berghei* rhomboid proteases, ROM4, 6, 7 and 8 were found to be essential, whereas ROM1, 3, 9, and 10 were dispensable for erythrocytic stage development (Lin 2013). The ROM3 knockout parasites developed normally until the oocyst stage but did not develop into sporozoites, indicating a crucial role of ROM3 in sporozoite development. On the other hand, knockouts of ROM9 and ROM10 did not affect any developmental stage, suggesting that these two proteases have redundant functions.

Threonine proteases

A threonine residue in these proteases mediates hydrolysis of the peptide bond in substrates. The best-known threonine proteases are the 26S proteasome complex and the mitochondrial ClpQY protease complex. Malaria parasites have both these protease complexes, and their inhibitors have been shown

to block parasite development, which supports their potential as drug targets (Reynolds 2007; Gantt 1998; Czesny 2009; Lindenthal 2005; Kreidenweiss 2008; Aminakea 2012; Jain 2013; Prasad 2013).

The proteasome

The proteasome is an integral component of the ubiquitin proteasome system (UPS), which is a major proteolytic machinery for degradation of damaged and disposable proteins in eukaryotes. The other integral component of the UPS is a ubiquitination component that specifically marks the target proteins for degradation with ubiquitin, and this component is composed of ubiquitin, the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2, and the ubiquitin ligase enzyme E3.

The ubiquitin-tagged proteins are degraded inside the proteasome. The proteasome is a large multiprotein complex that is organized into a 26S complex. A typical 26S proteasome complex is made up of two assemblies: a core protease complex, known as the core particle (CP) or 20S proteasome; and a regulatory unit, known as the 19S regulatory particle (RP) (Groll 1997). The CP is a barrel-shaped complex of four stacked rings arranged in αββα fashion, each with seven α (α1–7) or β (β1–7) subunits. Three catalytically distinct β subunits (β1, β2, and β5) are present in both β rings, and their active sites line the central lumen of CP to form a proteolytic chamber where substrates are degraded.

Substrates gain access to this chamber through the pores formed by α rings on either side of the CP (Baumeister 1998; Groll 2000), and entry requires the RP for opening the pores and unfolding of the substrate. The RP can associate with either end of the CP, and contains 19 proteins that are organized into two subcomplexes: the base and the lid (Glickman 1998). The base subcomplex opens the pore of CP, and it recruits and unfolds ubiquitinated proteins to the CP. The lid subcomplex deubiquitinates the recruited substrates before their degradation inside the proteolytic chamber. A typical 26S proteasome possesses three types of activities (Heinemayer 1997; Wilk 1980, 1983): caspase-like or peptidyl-glutamyl peptide hydrolyzing (PGPH) activity, which cleaves after acidic residues and is mediated by β1; trypsin-like activity that is mediated by β2 and cleaves after basic residues; and chymotrypsin-like activity of β5, which cleaves after large hydrophobic residues.

All studied *Plasmodium* species seem to contain a typical 26S proteasome complex (Aminakea 2012). Similar to the β1, β2, and β5 protease subunits of human and yeast, conserved domain analysis predicted Pfβ1, Pfβ2, and Pfβ5 to be threonine proteases with Thr and Lys residues that have been identified as essential catalytic residues in *Tthermoplasma acidophilum* β subunit and *Escherichia coli* threonine protease HslV. Consistent with antiparasitic effects of proteasome inhibitors on all developmental stages, multiple lines of evidence indicate the presence of proteasomes in all parasite stages (Aminakea 2012).

Researchers are encouraged by the approval of the proteasome inhibitor bortezomib for the treatment of multiple myeloma in humans, and the proteasomes of several parasites, including the malaria parasite, are being targeted for drug development. However, host toxicity of proteasome inhibitors has been a concern, and it could be a challenge to develop inhibitors specific for the parasite proteasome. Nonetheless, differences between the sequences of *Plasmodium* and host proteasome subunits may offer an opportunity to design specific inhibitors. In this context, proof of concept for designing a parasite proteasome-specific inhibitor was obtained with an analogue of the approved proteasome inhibitor carfilzomib (Li 2012), which showed strong antiparasitic activity *in vitro* and *in vivo*, without apparent toxicity to mammalian cells. This compound did not inhibit the β2 subunit of the human proteasome, perhaps explaining its lack of toxicity. In a recent study, Li 2016 designed a peptide inhibitor (WLL-vs) based on the differences in substrate specificity and structures of human and *P. falciparum* proteasomes. WLL-vs inhibited the activity of *P. falciparum* proteasome, but not that of human proteasome, and showed potent antimalarial activity both in *in vitro* and *in vivo* models of malaria infection, thereby provided a stronger evidence for targeting the parasite proteasome for development of antimalarials.

Roles of proteases in parasite development

The proteolytic repertoire of malaria parasites is likely involved in a large number of processes during parasite development. A large body of evidence from chemical inhibition of proteases and genetic studies unambiguously identifies critical roles of certain proteases in invasion of erythrocyte by merozoites, hemoglobin degradation during erythrocytic stage development, egress of merozoites from erythrocytes, and protein export, which are described below and/or in the sections dedicated to individual proteases.

Parasite egress and invasion of host cells

The full development cycle of malaria parasites involves multiple invasion and egress events, with invasion of hepatocytes by sporozoites, erythrocytes by merozoites, and the mosquito midgut by ookinetes and subsequent egress from hepatocytes, erythrocytes, and oocysts.

Sporozoites and merozoites are released upon rupture of the infected host cell, and this process is known as egress. Both egress and invasion have been most studied in the erythrocytic cycle, and both of these processes involve a cascade of proteolytic events. The current understanding of egress is based on studies using microscopy, protease inhibitors, and genetic manipulation of parasites.

Egress involves protease-mediated rupture of the parasitophorous vacuole and then erythrocyte membranes (Delplace 1988; Hadley 1983; Lyon 1986; Salmon 2001; Wickham 2003; Soni 2005; Glushakova 2005). Reports support a central role of serine and cysteine proteases in egress, with DPAP3 mediating maturation of PfSUB1, which then activates SERA5 and most likely other SERA proteins, and activated SERA5 possibly cleaving proteins both in the parasitophorous vacuole and erythrocyte membranes (Arastu-Kapur 2008; Yeoh 2007). The blocking of schizont rupture in the presence of anti-SERA5 antibodies supports a role for SERA5 in egress (Pang 1999). Knockout studies in *P. berghei* demonstrated that PbSERA5 (an orthologue of PfSERA8) knockout parasites had a block in egress of sporozoites from oocysts (Aly 2005).

What exactly degrades the parasitophorous vacuole membrane and erythrocyte cytoskeleton during rupture is not clear, but both parasite and host proteases may contribute to this process. E64 and leupeptin, two inhibitors of papain-like cysteine proteases, blocked merozoite egress from erythrocytes containing mature schizonts of *P. falciparum* (Hadley 1983; Lyon 1986; Salmon 2001; Wickham 2003); in agreement with this result, recombinant FP2/3 subfamily proteases have also been shown to degrade erythrocyte cytoskeleton proteins *in vitro* (Prasad 2012; Na 2004;Dua 2001), suggesting a role of these proteases in egress. Degradation of erythrocyte cytoskeleton by recombinant plasmepsin II suggests that it may also play a role in egress (Le 1999). However, gene knockout studies ruled out specific roles of FP1, FP2A, FP2B, and plasmepsins in egress (Bonilla 2007; Liu 2005; Sijwali 2004). These results could be due to functional redundancies in the proteolytic repertoire of malaria parasites.

Considering host proteases, erythrocyte calpain has been shown to be crucial for merozoite egress, as its specific inhibition or immunodepletion blocked rupture of schizont-infected erythrocytes (Chandramohandas 2009). Although the sequential events of egress from erythrocytic merozoites and the specific roles of proteases have not been fully established, extensive studies support crucial roles for both parasite and host proteases in egress.

Protease inhibitors have been extensively used to study invasion of erythrocytes by merozoites. Failure of merozoites to invade erythrocytes in the presence of the serine protease inhibitor chymostatin suggested a role of serine proteases in invasion (Hadley 1983; Breton 1992). Studies with inhibitors of other classes of proteases, including E64 and pepstatin, have yielded mixed results, but the majority of studies rule out roles of cysteine and aspartic proteases in invasion (Hadley 1983; Banyal 1981; Dluzewski 1986; Breton 1992).

Studies employing chemical inhibition of proteases, gene knockouts, and cell biology techniques, indicate crucial roles of subtilisins and rhomboid proteases in invasion. Invasion of erythrocytes by merozoites involves interactions between several parasite and erythrocyte proteins, and the final interacting domains of parasite proteins are generated as a result of site-specific cleavages by subtilisins. PfSUB1 has been shown to processes multiple merozoite surface proteins (MSP1, MSP6, and MSP7), which primes merozoites for invasion of erythrocytes (Koussis 2009). It has been suggested that PfSUB2 processes the MSP1 42-kDa fragment, leaving the membrane-bound MSP1 19-kDa fragment on the merozoite surface (Barale 1999). The recombinant PfSUB2 propeptide, a potent inhibitor of mature PfSUB2, selectively reduced shedding of MSP1 and AMA1, indicating a role of PfSUB2 in maturation of these two antigens (Harris 2005).

Unsuccessful knockout of the *P. berghei* subtilisin-2 gene supports an essential function of SUB2 in erythrocytic parasite biology (Uzureau 2004). A number of *Plasmodium* adhesins, proteins that mediate adhesion to host cells, have been reported to be shed as a result of cleavage within or near their transmembrane domains, and several rhomboid proteases have been implicated in this process. Some key adhesins have been mentioned above. ROM1 cleaves AMA1 (Baker 2006) and ROM4 EBA175 in *P. falciparum* (O'Donnell 2006). ROM4 also cleaves the TMD of the major sporozoite adhesin TRAP *in vitro,* and mutations within the cleavage site abrogated the shedding, resulting in a significant loss of infectivity *in vivo* (Baker 2006; Ejigiri 2012).

Because blocking the shedding and/or maturation of parasite proteins blocks invasion, simultaneous inhibition of both processes will likely result in potent antiparasitic effects. Hence, both subtilisins and rhomboid proteases are considered attractive drug targets.

Hemoglobin degradation

Hemoglobin degradation is probably the most extensively studied proteolytic process in malaria parasites. Hemoglobin makes up 33% of the volume of erythrocyte cytosol and 98% of cytosolic protein, and malaria parasites degrades 60% to 80% of erythrocyte hemoglobin in the food vacuole over the course of the erythrocytic cycle. Hemoglobin degradation is required to obtain amino acids and probably to maintain osmotic stability of the infected cell. A number of proteases mediate this vital catabolic process (Ball 1948; Morrison 1948; Orjih 1993; Rosenthal 2005; Goldberg 2005; Lew 2003; Krugliak 2002; Shermann 1977). The process of hemoglobin uptake is not clearly understood; microscopy studies indicate its uptake through a cytostome-like organelle from which hemoglobin-containing vesicles bud off and deliver their contents to the food vacuole (Francis 1997). Hemoglobin degradation releases heme and amino acids; heme is polymerized into a nontoxic pigment, known as hemozoin (Krugliak 2002; Shermann 1977).

Early studies using the protease inhibitors E64 and pepstatin suggested that cysteine and aspartic proteases degrade hemoglobin (Dluzewski 1986; Shermann 1977; Rosenthal 1988; Bailly 1992). Treatment of parasites with E64 or synthetic cysteine protease inhibitors both in culture and in animal models blocked parasite development, with accumulation of undegraded hemoglobin in the food vacuole, which indicated a key role of falcipains in hemoglobin degradation (Rosenthal 1988; Rosenthal 1993; Rosenthal 1989). A number of biochemical and cell biology studies have shown that four falcipains and four plasmepsins are present in the food vacuole, maximally active at the food vacuole pH, and degrade hemoglobin, indicating that both falcipains and plasmepsins degrade hemoglobin in the food vacuole (Banerjee 2002; Goldberg 1991; Shenai 2000; Sijwali 2001; Rosenthal 2009, 1988, 1987; Singh 2006; Bailly 1991; Goldberg 1990).

Gene-knockout studies have confirmed a critical role of FP2A in hemoglobin degradation, whereas knockouts of all four plasmepsins, FP1, and FP2B did not cause any noticeable effect on hemoglobin hydrolysis (Bonilla 2007a, 2007b; Liu 2005; Sijwali 2004a, 2004b; Sijwali 2006). FP2A is most abundantly expressed in the trophozoite stage, and it contributes most of the total cysteine protease

activity in that stage (Shenai 2000; Sijwali 2001), when hemoglobin degradation is maximal. FP2A knockout parasites showed decreased hemoglobin hydrolysis, accompanied by enlargement of the food vacuole (Sijwali 2004b), further supporting the conclusion that FP2A has a major role in hemoglobin hydrolysis during the trophozoite stage. However, the food vacuole phenotype of FP2A knockout parasites disappeared during late trophozoite and schizont stages, indicating normal hemoglobin hydrolysis. The recovery of FP2A knockout parasites is probably due to involvement of other proteases, most likely FP3, which is predominantly expressed in late trophozoite and schizont stages and appears to be essential for parasite development (Sijwali 2001; Sijwali 2006). Compared to wild-type parasites, FP2A knockout parasites also showed two-fold greater sensitivity to cysteine protease inhibitors and almost 1000-fold greater sensitivity to the aspartic protease inhibitor pepstatin (Sijwali 2004b; Sijwali 2006; Liu 2006), supporting collaborative roles of falcipains and plasmepsins in hemoglobin hydrolysis (Semenov 1998).

Falcipains have also been shown to be required for maturation of the food vacuole plasmepsins (Drew 2008), consistent with the extreme susceptibility of FP2A knockout parasites to pepstatin. Thus, as the knockout of FP2A and treatment of parasites with E64 resulted in the same phenotype, with swelling of the food vacuole and accumulation of undegraded hemoglobin, FP2A and most likely FP3 are the principal hemoglobin-degrading cysteine proteases. Although knockouts of plasmepsins or treatment of parasites with pepstatin did not have any major effect on hemoglobin hydrolysis, an accessory role of plasmepsins in this process seems likely.

Hemoglobin hydrolysis by falcipains and plasmepsins would produce oligopeptides, which are further degraded into smaller peptides and amino acids by oligopeptidases and aminopeptidases

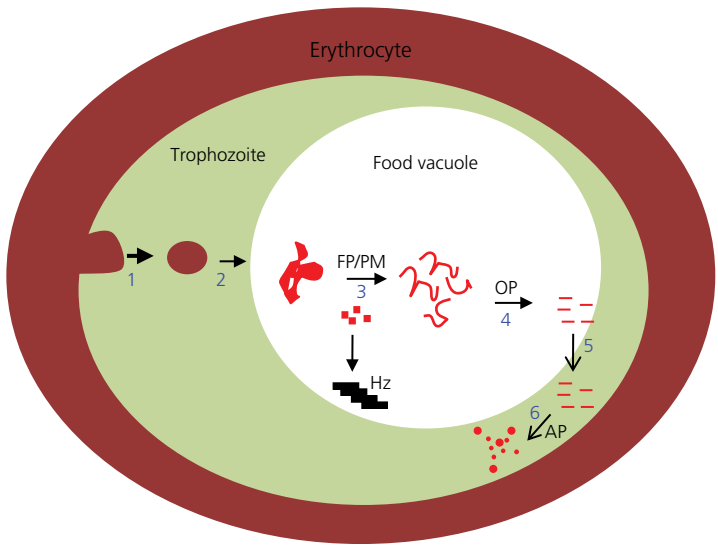

Figure 12.6 Schematic of hemoglobin degradation. Shown is a cartoon of an erythrocyte infected with the trophozoite stage, which voraciously degrades hemoglobin in the food vacuole. The parasite takes up the erythrocyte cytosol through a cytostome-like organelle (1) wherefrom vesicles bud off (2) and transport hemoglobin to the food vacuole. Hemoglobin appears to be first degraded (3) by falcipains and plasmepsins (FP/PM) that generate oligopeptides, which are further degraded (4) by oligopeptidases (OP: falcilysin, PfA-M1, and DPAP1) into smaller peptides. The smaller peptides have been proposed to be transported into the parasite cytosol (5), where aminopeptidases (AP: PfLAP and PfAP) degrade these into free amino acids, which are used by the parasite. Hemoglobin degradation (3) results release of free heme, which is polymerized into a nontoxic polymer, known as hemozoin (Hz).

(Gavigan 2001). The oligopeptidases include falcilysin and DPAP1; the aminopeptidases include PfA-M1 (Klemba 2004; Ragheb 2011; Harbutt 2011; Eggleson 1999). These three proteases have also been shown to be present in the food vacuole and degrade hemoglobin oligopeptides at acidic pH, supporting their role downstream of falcipains and plasmepsins in hemoglobin degradation. It has been proposed that these small peptides are transported out of the food vacuole into the cytoplasm, where cytosolic aminopeptidases, including PfLAP and PfAAP (Stack 2007; Gardiner 2006; Teusher 2007), generate free amino acids.

Thus, multiple lines of evidence indicate that multiple proteases participate in hemoglobin hydrolysis, in which falcipains most likely function as the principal proteases and plasmepsins as accessory proteases on hemoglobin to generate oligopeptides; falcilysin, DPAP1, and PfA-M1 act on oligopeptides to generate smaller peptides that are transported into the cytoplasm; and PfLAP and PfAAP act at the final stage of the degradation pathway to generate free amino acids (Figure 12.6). Of interest, potent activity of artemisinins, the mainstay of modern treatment for falciparum malaria, requires falcipain activity (Klonis 2011); thus, falcipain inhibitors should probably not be combined with artemisinins to treat malaria. In contrast, inhibitors of cysteine and aspartic proteases showed synergistic antimalarial effects, consistent with a complementary role for these two classes of enzymes, and suggesting the potential for synergistic combination antimalarial therapy (Semenov 1998).

Summary and conclusions

The presence of a large repertoire of diverse proteases in malaria parasites suggests multiple roles for these enzymes during parasite development. Because only a handful of these proteases have been biochemically characterized, and because only a few of the biochemically characterized proteases have been definitively assigned biological functions, much additional work is needed to understand the roles of the proteolytic repertoire in parasite development. Because proteases are considered attractive drug targets for a number of diseases, the large proteolytic repertoire of malaria parasites presents a treasure of drug targets. In fact, a number of proteases, including falcipains, plasmepsins, DPAPs, metalloproteases, and subtilisins, have already been validated as drug targets, and a number of drug discovery projects are under way to develop their specific drug-like inhibitors (Rosenthal 2011; Ersmark 2006; Withers-Martinez 2004; Skinner-Adams 2008; McKerrow 2008; Arastu-Kapur 2008; Wang 2011; Harbut 2011; Eggleson 1999; Bouillon 2013; Gelb 2007; Linares 2007; Meyers 2012; Cteron 2010). The availability of structures of some of these proteases should aid in the development of parasite protease-specific inhibitors as antimalarials.

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CHAPTER 13

Development of medicines for the control and elimination of malaria

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Medicines have been used to treat malaria for centuries: Artemisinin from the Qinghao plant was used in 4th-century China, and quinine from the cinchona tree was used in 17th-century South America. In the 21st century, uncomplicated malaria caused by *Plasmodium falciparum* can be cured in 99.9% of cases when it is promptly and appropriately treated with any of a number of effective medicines that now exist (Fauci 2011). Despite this, malaria is still a significant global burden in terms of morbidity and mortality (World Health Organization 2015b) because the development and deployment of acceptable and effective medicines for the control and elimination of malaria is about so much more than pharmacology.

Communities affected by malaria are often poorly resourced in terms of clinical infrastructure, education, and finances and may be resistant to change from long-established but no longer appropriate treatment-seeking behaviors. Even patients who are able to access potentially effective medicines might not adhere to the treatment regimen, rendering the treatment ineffective in the short term and vulnerable to the development of resistance in the long term.

The malaria parasite, in common with other infective agents, has the potential to mutate, developing strains that are resistant to antimalarial drugs, as was often seen in the past. This means that in some areas, drugs that were once effective have become ineffective, or at least less effective, in clearing parasites from the patient's blood. Some medicines are more vulnerable to the emergence of resistance than others, and some parts of the world (particularly the Thailand–Cambodia border region) appear more prone to the appearance of drug-resistant parasites than others.

The development of resistance can be slowed by making sure that all treatments used are combination therapies by only treating confirmed cases of malaria with an appropriate dosage regimen and by the instigation of monitoring programs to track resistance (World Health Organization 2015a).

The historical experience of drugs being quickly rendered ineffective by the development of resistant parasites has led researchers to design therapies specifically to protect their efficacy, either by combining drugs with different mechanisms of action or by looking for new classes of drugs altogether.

Babies, young children, and pregnant women are among the most vulnerable to malaria. Understanding the safety of new medicines is always a priority, and consequently all have to be proved effective and safe on adult populations first. Child-friendly formulations are often only developed later, but these are a priority because taste masking and easily dispersible tablets are key to increasing compliance with new therapies. Malaria patients who are co-infected with HIV (as occurs commonly) require treatment that is safe and effective both in combination with

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antiretroviral drugs and with the underlying immune suppression that comes with the disease. These special needs are not always easy to accommodate in the early stages of the drug-development process.

In recent years there has been an increase in investment. More than $100 million a year is invested in developing and delivering medicines with the potential to be acceptable and effective in all malaria patients. This is still only a tiny fraction of the overall investment in parasitology, with the principal focus of the community still being on basic science and vaccine design. The significant reduction in cases of malaria in some parts of the world has led communities to look beyond management and control of malaria toward the long-term goal of elimination. The ideal medicine for use in these circumstances has specific properties: rapidly killing the parasite (with a simplified dose or preferably a single dose of therapy) and preventing relapse of dormant forms and the transmission of the parasite. These "ideal" characteristics present additional challenges to the drug-development community.

Historical treatments for malaria

Malaria has affected humans in various parts of the world for thousands of years, and for hundreds of years it has been recognized that some plants have antimalarial properties. The bark of the cinchona tree native to South America was brought to Europe as the "Jesuit's bark" (Cook 2014) in the 1600s as a cure for malaria. In 1820, two French chemists isolated quinine from the cinchona bark, and quinine became a treatment of reference for intermittent fever throughout the world (Guerra 1977a, 1977b). It remains an important and effective treatment for some populations affected by malaria today, although seven days of treatment three times per day are required for it to be effective. *Artemisia annua* (sweet wormwood), known to Chinese herbalists for more than 2000 years as "Qinghao", received the attention of Chinese and then European scientists from 1970 onward. They extracted the active compound artemisinin and developed the class of drug that has become the main line of defense against malaria throughout Africa and South East Asia. Youyou Tu's work in China played a key role in this discovery and in 2015 she shared the Nobel Prize for Medicine in recognition for these efforts (Van Voorhis 2015). These molecules are the foundation of the artemisinin combination therapies (ACTs), which have allowed the use of three-day treatment regimens.

In the 1930s, German scientists looking for a substitute for quinine synthesized two compounds: resochin (chloroquine) and sontochin (3-methyl-chloroquine). Following the Second World War, chloroquine and the insecticide DDT became the mainstay of the World Health Organization's (WHO) global eradication malaria campaign. Resistance to chloroquine emerged in *P. falciparum* parasites on the Thailand–Cambodian border around 1957 and spread to most parts of the world by the 1980s, rendering chloroquine ineffective. Other derivatives of 4-aminoquinolines, such as amodiaquine and piperaquine, are still in widespread use today, but as part of artemisinin-combination therapies.

Mefloquine, an aminoalcohol derived from quinine by the US Army at the time of the Vietnam war, has not been adopted as widely as other medicines, and it has been used primarily in prophylaxis. Again originating in the Thailand–Cambodia border region, parasite resistance to mefloquine is spreading. Lumefantrine, a related aminoalcohol developed by Chinese scientists at around the same time as mefloquine, has become the mainstay of the most widely used combination therapy, and so far it has not seen significant resistance, largely because it has never been used clinically as a monotherapy.

Proguanil, its metabolite cycloguanil, and the related pyrimethamine (both anti-folates) were developed as antimalarials around the same period. However, the rapid development of resistance through mutation of the target, *Plasmodium* dihydrofolate synthase (DHFR), led to the combination of these drugs with sulfones and sulfonamides, which target a different enzyme, *Plasmodium*

dihydropteroate synthase (DHPS), in the hope of increasing their usefulness. However, resistance still quickly developed to sulfadoxine–pyrimethamine in Thailand, and this eventually spread to Africa in the late 1990s.

This history of single antimalarial drugs rapidly becoming ineffective when used in malaria-endemic communities has led to the development of combination therapies. Compounds with different modes of action are combined to maximize their therapeutic properties and protect them from the development of resistance. In the very rare event that a mutant parasite resistant to one of the medicines arises *de novo* during the course of the infection, this resistant parasite will be killed by the other antimalarial medicine. This mutual protection is thought to prevent or to delay the emergence of transmissible clinical resistance (World Health Organization 2015a). In addition, if the two molecules have pharmacological synergy, it is possible to use a lower dose to achieve the desired efficacy, and this helps to reduce cost and side effects.

Artemisinin combination therapies

The current WHO-recommended first-line treatment for uncomplicated malaria cases is artemisinin-based combination therapy (World Health Organization 2015a). These are medicines that combine an artemisinin derivative (for example, artemether, artesunate, or dihydroartemisinin) with a partner drug. Artemisinin derivatives are peroxides that are active against all species of *Plasmodium* and able to rapidly kill the blood stages of the parasite and reduce fever quickly (World Health Organization 2015b). Combining artemisinin with a partner drug in one tablet (a fixed-dose combination) is preferred, because it ensures that both active ingredients are taken at the right dosages, maximizing efficacy and reducing the potential for resistance developing. Anecdotes from the field strongly suggest that when certain combinations are packaged as a co-blister, the patients know that most of the side effects are caused by the partner drug and therefore often take only artemisinin monotherapy.

Six fixed-dose ACTs have been reviewed by stringent regulatory authorities and/or prequalified for supply by the World Health Organization throughout the world: artemether–lumefantrine (Coartem and Coartem Dispersible from Novartis), artesunate–amodiaquine (Coarsucam/ASAQ-Winthrop from Sanofi), dihydroartemisinin (DHA)–piperaquine (PPQ; Eurartesim from sigma-tau), pyronaridine–artesunate (Pyramax from Shin Poong), artemisinin–naphthoquine (ARCO, Kunming, China), artesunate–mefloquine (developed by DND*i* (the Drugs for Neglected Diseases *initiative*) initially with Farmanguinhos in Brazil, and now with Cipla in India, as well as a Cephalon/Mepha combination available in Africa).

In a move to protect artemisinin from the development of resistance, WHO has recommended that oral artemisinin-based monotherapies be withdrawn from the market. Unfortunately, these monotherapies are still available in many malaria-endemic countries, and this could be hastening the development of resistance to artemisinins.

ACTs are efficacious and safe antimalarials. However, they still have several characteristics that could be improved on. First, they require a three-day course to be effective. In many communities, correct adherence by patients or caregivers to the dosing regimen is not guaranteed, increasing the risk of resistance developing. Fever is normally cleared in the first two days, leading patients to discontinue therapy early. In areas of high transmission, people may experience as many as ten malaria episodes per year, increasing the temptation to retain part of the medicine course "for the next time" or for another child in the family. The other issues are that ACTs do not prevent the relapse of dormant forms of *Plasmodium vivax* and *P. ovale* and that they do not completely block the transmission of the parasite to new mosquitoes.

Within the six fixed-dose ACTs currently recommended, some of the properties of the partner drugs make them less desirable than others in certain populations. For example, amodiaquine has

resistance problems, mefloquine has tolerability issues, and there is insufficient information to allow DHA–PPQ for use as first-line therapy in pregnancy (Tansley 2010). Lumefantrine, the partner drug in the most widely used ACT, is part of a six-dose treatment and has the theoretical disadvantage of maximum absorption being highly dependent on intake with fatty foods. Studies have, however, shown that the fat content of standard meals or breast milk in sub-Saharan Africa is adequate to achieve optimal efficacy for this combination (Premji 2008).

One particular challenge with small children is the risk that the bitter taste of the medicine causes them to spit it out, with the result that they do not receive a full dose. In addition, some medicines have to be crushed before being suspended in liquids for administration to children, and this also risks that a child will not get a complete course of treatment. To overcome these two issues, a sweetened cherry-flavored, easily dispersible form of artemether–lumefantrine (Coartem Dispersible) was produced, in a collaboration between Medicines for Malaria Venture (MMV) and Novartis, and launched in early 2008. Almost 50 million children are now treated with this child-friendly medicine each year. Although several companies (such as Cipla and Ipca in India) make generic artemether–lumefantrine in the same price range, Novartis is still the only producer of the pediatric form. In 2015, the European Medicines Agency (EMA)approved Pyramax, a granule formulation of pyronaridine–artesunate, for pediatric use. It was developed by MMV and Shin Poong, and, following successful trials (Sagara 2015) it was approved under Article 58, on products that are not intended for use in Europe. One other ACT pediatric formulation is in development: a dispersible formulation of DHA–piperaquine. The alternative option for children, crushed tablets, is likely to be less acceptable than a sweet, flavored liquid and risks inaccurate dosing in this vulnerable group.

Finally, all artemisinin-based drugs rely on the supply of the raw material from the *Artemisia annua* plant, because complete chemical synthesis is far too costly. This situation places an enormous strain on the supply chain because material can take up to two years from order to delivery (the plant is slow growing), and the price fluctuates massively; over the last few years it has varied between $300 and $1,000 per kilogram. In recent years, MMV established a program introducing greater cooperation and coordination within the industry, enabling all stakeholders involved in the cultivation, extraction, and derivatization of artemisinin to identify ways they could reduce costs and increase efficiency. This program is becoming superseded by new technologies that allow the production of the precursor of artemisinin, artemisinic acid, in yeast (Paddon 2013) on a ton scale, allowing stable production of artemisinin at around $400 per kilogram. Although this is not significantly cheaper than the mean price of artemisinin, it does mean that the fluctuating peak prices should become a thing of the past.

MMV has also launched a project with the University of Nebraska, the Swiss Tropical Institute, and Monash University in Australia to develop synthetic peroxides. This has resulted in two success stories: OZ277 (now called Arterolane or Rbx11160), which was launched in India as a combination called Synriam by Ranbaxy under license from MMV in 2012 (Toure 2016;Valecha 2016), and a second-generation compound, OZ439, that has recently completed Phase IIa studies and is discussed below.

Targets for the development of future medicines for malaria

In the development of new treatments for malaria, a number of key generic criteria need to be met by any drug candidates: high clinical efficacy (*i.e.*, a cure rate 28 days after taking the medicine of greater than 95%), low cost (less than US$0.25 for a pediatric course of treatment), a low

potential for the emergence of clinically significant resistance, and good tolerance in the target populations. Any new medicines will be compared against the current gold standard of ACTs. The constant threat of emergence of resistance can be counterbalanced by testing against current clinical isolates, measuring the ease with which resistant mutants can be raised *in vitro,* and always choosing combinations that affect different pathways in the parasite (Ding 2012). Patient safety is of paramount importance: Current medications have extremely rare serious adverse events (less than one in a thousand), and new medicines or vaccines would be expected to improve on even this target.

Many countries have begun to discuss eliminating malaria. In the near term, an increased number of individuals could receive drugs for malaria chemoprevention rather than treatment (in pregnancy this is known as IPTP, or intermittent preventive treatment in pregnancy; in small children this is known as seasonal malaria chemoprevention or SMC). These strategies are extremely effective, causing a much larger decrease in mortality than vaccines, at a fraction of the price. However, new medicines for protection, (and new vaccines) would have to be even safer than medicines used for treatment, further raising the bar for any new medicine.

Drug development and the elimination agenda

Treating patients who have malaria symptoms will not be enough to eliminate the disease. There are two additional reservoirs that need targeting. After infection, patients have malaria gametocytes in their blood (these are the form of parasite transmitted to mosquitoes with a blood meal). Drugs that are effective against both gametocytes and asexual forms are therefore a priority. In addition, there is a need for new molecules that target the dormant liver stages of *P. vivax* and *P. ovale*. Unless these dormant parasites are killed, they can become reactivated, causing disease relapse and a new episode of malaria without a mosquito bite. The current treatment for these relapses is primaquine (White 2013), which has two problems: It has to be given for 14 days, so compliance is poor (especially since patients are asymptomatic and may suffer gastrointestinal side effects), and it is contraindicated in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Unfortunately G6PD deficiency co-segregates with malaria endemicity and in many places represents 10% to 15% of the population (Cappellini and Fiorelli 2008). Furthermore, since the G6PD status of a fetus is unknown, primaquine is contraindicated for pregnant women. Figure 13.1 depicts the growth and development of medicines for malaria control and elimination.

The importance of partnerships in drug discovery and development

The discovery and development of new medicines against malaria represents a formidable challenge. The lack of an obvious financial return on investment (the profits made by selling new medicines will not repay the development costs) is a good example of market failure. However, it has also resulted in a scientific environment of highly collaborative interaction between academia and the pharmaceuticals industry, with groups sharing data in ways that would be unthinkable in more-commercial therapeutic areas. The addition of financial support from government development agencies and philanthropic agencies to the massive in-kind support from companies and universities has enabled this market failure to be overcome in many (but not all) cases. Malaria-endemic communities play an essential role in enabling the design and implementation of clinical trials. More recently we have seen the emergence of new medicines (clinical candidates) from projects run in disease-endemic countries such as Thailand and South Africa. This cooperation has transformed the malaria landscape and holds the key to success in the development of effective treatments and preventive strategies for the future.

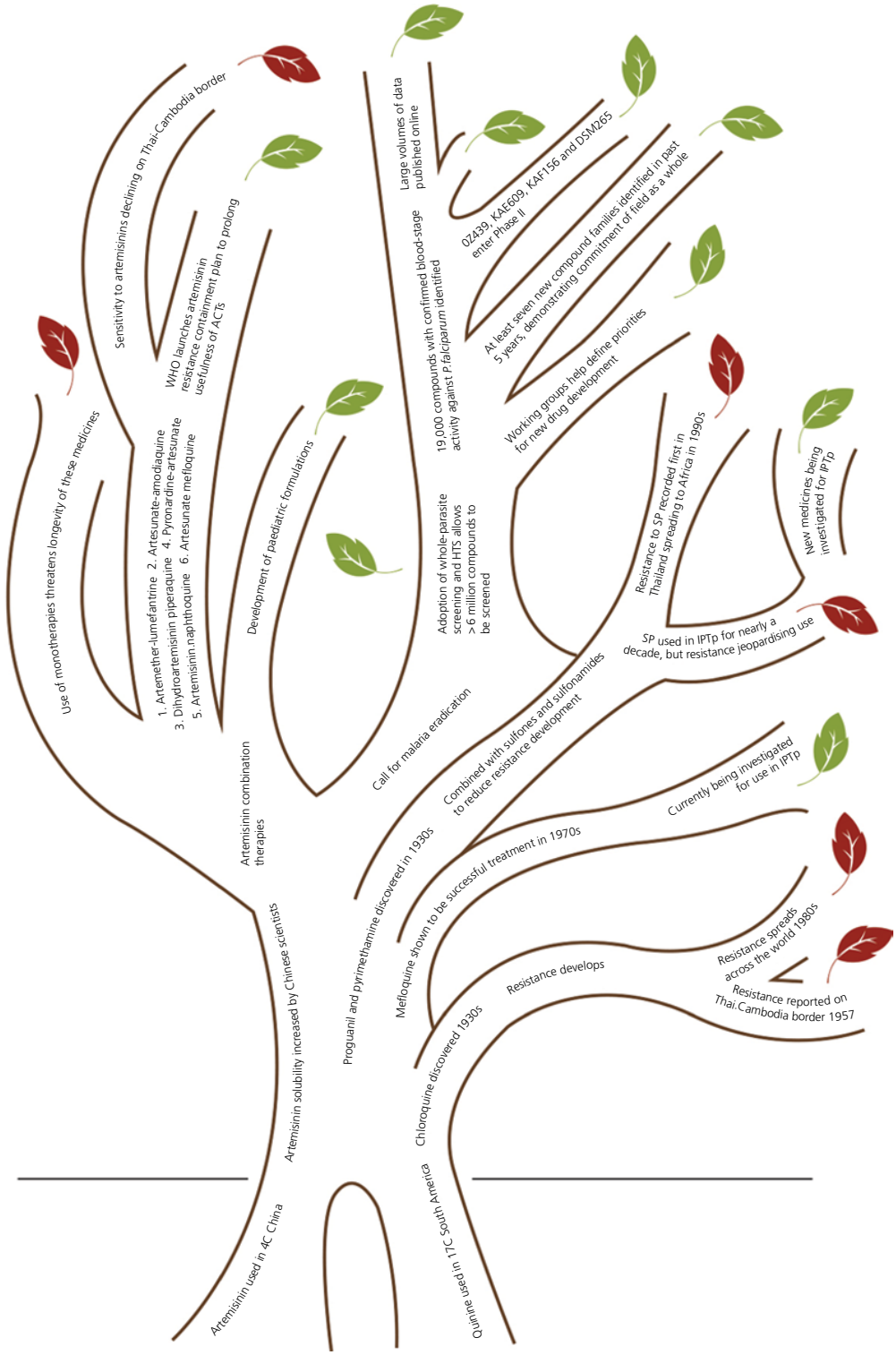

Figure 13.1 The growth and development of medicines for malaria control and elimination.

The process of drug development

Following the call in 2007 for a program to eradicate malaria, the elaboration of this agenda by various working groups has helped to identify priorities for the future (Alonso 2011). The eradication agenda outlines the requirement for new medicines that are suitable for elimination-specific indications such as mass treatment, curing asymptomatic infections, curing relapsing liver stages, and preventing transmission (malERA Consultative Group on Drugs 2011). The delivery of these new medicines will require the identification and development of new molecules with specific properties. To successfully identify and deliver these medicines, a major input of specialized research, technical expertise, and the development of quality assays will be necessary. Leveraging the combined facilities, knowledge, resources, and experience of all players in the malaria community, including industry, academia, nongovernmental organizations and donors, will be crucial.

As described in the introduction, ACTs have become the cornerstone of malaria-control programs, treating acute disease and preventing complications in the most vulnerable populations. These drugs will continue to play an important role in the journey toward eradication because they will remain critical for case management. However, with a move toward the ultimate goal of malaria eradication, the priorities for new drugs are much broader. For malaria eradication there is a need for medicines that can be used as a single dose, and these medicines need to contain potent molecules with different mechanisms of action and routes to generating resistance. In addition, new medicines must kill gametocytes, which are responsible for transmission, and the hypnozoites of *P. vivax*, which cause relapse. Finally, exceptionally safe drugs with long half-lives will be required for chemoprevention indications. These characteristics are described by target product profiles (TPPs), which give a description of the ideal medicine and the minimum acceptable profile.

To ensure that the next generation of drugs meets the new eradication-specific challenges, two TPPs (currently under review) have been developed that outline the requirements for these new antimalarials.

- TTP1: combination blood-stage cure, with transmission blocking, hypnozoiticidal, and post-treatment prophylactic action
- TTP2: single-exposure chemopreventive drug

With safe and efficacious products already on the market, it is no longer sufficient to deliver a multiple-dose blood-stage treatment unless the mainstay therapies are lost to resistance or adverse events. The ideal new medicine would eliminate the human reservoir of parasites, provide long-lasting protection from reinfection, and reduce the course of treatment to a single dose (single-exposure radical cure and prophylaxis; SERCaP). Such a medicine would transform drug treatment for malaria. The TPPs provide drug discoverers with a common standard of the unmet clinical needs in malaria control and eradication, thereby allowing a better focus.

Combination medicines

As outlined earlier, fixed-dose combination medicines are now recommended by WHO for the treatment of malaria, because they tend to be both more effective and more resistant to the development of resistance. Consequently any new medicine developed should be a combination of at least two complementary molecules. Following consultations with its partners, MMV has defined four target candidate profiles (TCPs; Burrows 2013), which aim to guide the identification of molecules that might be combined to produce a medicine that meets the TPPs described above. The four TCPs are

- TCP1: Rapid-parasite-clearance molecules that could replace artemisinin
- TCP2: Molecules with long half-lives to give post-treatment prophylaxis

- TCP3: Molecules that kill nondividing parasite forms: hypnozoites, to stop *P. vivax* relapse (which we have termed TCP3a) and gametocytes, to stop transmission (termed TP3b)
- TCP4: Slow-onset chemoprophylactics or causal prophylactics with long duration and excellent safety profiles to protect vulnerable populations in once-endemic regions

The development of a combination medicine for malaria requires the consideration of a number of specific factors. For example, it is important that both partners have a high potency; two low-potency drugs would not produce a useful medicine owing to large pill size and potentially the high cost of goods. Many of the current antimalarial treatments are administered at high dosage levels. Ideally, new medicines would have a high potency and improved pharmacokinetic parameters that would allow much lower dosage. This has the added benefit of keeping costs as low as possible. New medicines would need to be provided to malaria-endemic countries at a price that is equivalent to, or lower than, the ACTs currently in use. This sets an upper limit on the cost of any new treatments.

Pairing compounds with the same mechanism of resistance development, similar safety liabilities, or molecules that share the same metabolic or excretion pathway should be avoided. In an ideal combination, the compounds would work synergistically in terms of exposure and efficacy, and both drugs should meet their TCPs and deliver the TPPs together; the compensation of deficits of one drug by the other, for example with resistance liability, is not an acceptable strategy because it makes the partner drug and combination more vulnerable. Combining partner drugs with different speeds and durations of action can produce medicines that are suited to use in different scenarios. The different types of medicine that could be produced are outlined in Table 13.1. The other major consideration when producing a combination medicine is at which point during the drug development process combination studies should begin. This will be considered in more detail in a later section of this chapter.

Identifying new antimalarial compounds

The first stage in developing a new medicine for malaria is the identification of compounds with antimalarial properties *in vitro*. The rapid increase in the availability of genome data was proposed as a mechanism to accelerate the discovery of new antimalarials by speeding up target validation. Unfortunately, these molecular-based approaches have not been particularly efficient in infectious diseases (Payne 2007). The reasons are complex, but genetic validation does not always correlate with drug responses *in vivo*. In addition, genome-based approaches tend to be biased by what is already known, and the focus was perhaps too much on targets with well-known pedigrees such as kinases, phosphatases, and proteases.

For parasitic diseases, (phenotypic, or high-content) screening can be carried out directly against the whole parasite, and this has proved extremely fruitful. A key advantage of parasite-based screening is that active compounds may have more than one molecular target; such polypharmacology would protect compounds against the development of resistance (Ding 2012). Changes in technology since the turn of the century have reduced *Plasmodium* screening costs by more than 100-fold and have increased throughput by a similar amount, meaning that screening hundreds of thousands of compounds rapidly is now possible.

Close to six million compounds have been screened against the erythrocytic stages of malaria, leading to the identification of more than 25,000 hits with submicromolar activity. GlaxoSmithKline, Novartis, and St. Jude Children's Research Hospital have released information on approximately 19,000 compounds (https://www.ebi.ac.uk/chembl/malaria/) to encourage drug lead identification efforts (Gamo 2010; Guiguemde 2010). The richness of the compound database means that the rate-determining step in drug discovery has now shifted to the downstream issues of lead optimization (making compounds safe, orally bioavailable, and efficacious *in vivo*) and working on the other

Table 13.1 Combining different types of blood-stage activities to produce a new medicine, with a summary of their advantages and disadvantages.

	Fast-acting Short-lasting	**Fast-acting Long-lasting**	**Slow-acting Short-lasting**	**Slow-acting Long-lasting**
Fast-acting Short-lasting	Rapid parasitemia reduction and symptom improvement Low risk of resistance induction Favorable safety profile Useful in severe malaria or travelers Re-infection possible Potentially multiple doses required	Rapid parasitemia reduction and symptom improvement Reduced risk of re-infection Some risk of resistance development to long-lasting partner	Repeat of earlier combination	Rapid parasitemia reduction possible if fast-acting partner has high parasite-reduction ratio[1] Some risk of resistance development to long-lasting partner
Fast-acting Long-lasting	Rapid parasitemia reduction and symptom improvement Some risk of resistance development to long-lasting partner	Rapid parasitemia reduction and symptom improvement Low risk of resistance induction or recrudescence Reduced risk of re-infection Reduced risk of resistance induction or recrudescence Potential safety issues related to prolonged exposure of two compounds	Repeat of earlier combination	Rapid parasitemia reduction possible if fast-acting partner has high parasite-reduction ratio[1] Low risk of resistance induction or recrudescence Reduced risk of re-infection Potential safety issues related to prolonged exposure of two compounds
Slow-acting Short-lasting	Rapid parasitemia reduction possible if fast-acting partner has high parasite-reduction ratio Potentially equivalent to monotherapy as no contribution of second drug Re-infection possible Risk of resistance induction Multiple doses required for efficacy	Rapid parasitemia reduction possible if fast-acting partner has high parasite-reduction ratio[1] Some risk of resistance development to long-lasting partner	No utility	No utility
Slow-acting Long-lasting	Repeat of earlier combination	Repeat of earlier combination	No utility	Good for chemoprevention indications Potential safety issues related to prolonged exposure of two compounds

important stages of the parasite lifecycle. Target-based screening is also enjoying a renaissance; the targets of the most advanced molecules coming from the whole-parasite screening programs have now been identified, and include two membrane channels, a kinase and an enzyme (Wells 2015). Interestingly, none of the "new targets" were on the list of high-priority targets coming from genomic sequences three or four years ago.

To make the malaria hits more accessible to the biology community, MMV has used cluster analysis to refine this list into a collection of 400 pragmatically selected, commercially available compounds. This compound set, called the Malaria Box (Spangenberg 2013), has been shipped to more than 250 groups all over the world. More than 35 papers have been published with screening results, disclosing hits against 16 different protozoa, seven helminths, nine bacterial and mycobacterial species, and *Aedes aegypti*, the mosquito that transmits chikungunya, Zika and dengue fever, and human cancer cell lines. A unique manuscript that describes these multiple groups' data, with more than 185 authors, is due for publication in 2016 in PLoS Pathogens (van Voorhis et al., *"Open-source drug discovery with the Malaria Box compound collection for neglected diseases and beyond"*). All data have been deposited in a ChEMBL website (https://www.ebi.ac.uk/chembl/malaria/).

The highly successful Malaria Box was succeeded by the Pathogen Box (http://www.pathogenbox.org/), launched in December 2015, which contains compounds that target a wider set of disease pathogens: *Tuberculosis, Plasmodium* and other kinetoplastids, helminths, *Cryptosporidium*, *Toxoplasma,* and dengue virus, as well as reference compounds. In collaboration with DND*i*, MMV is defining a new open-access compound box, the Pandemic Fever Box, focused on combating viruses and bacteria associated with pandemic response. This set is to comprise pathogen-targeting compounds from academic and industrial labs that were once in development but have since been abandoned.

These open source initiatives are based on a number of rationales. One is the widely accepted view that scientific discovery thrives best in an open environment. The second is to involve the dispersed community of research teams that focus on neglected pathogens in drug discovery, not only with access to high-quality screening tools but also with community-wide access to expertise (peer review) and standardized procedures. The specific goal of the Pandemic Response Box is, in addition, to shorten the time between the emergence of a new pandemic and the availability of new drugs against the responsible pathogen. History has shown time and again that in these situations, saving time saves lives. The box is meant to represent druggable aspects relevant to a wide set of pathogens, and it can be used to test for efficacy even before the pathogen's biology is fully understood.

The drug-development process

As described above, the development of a new medicine begins with the identification of compounds that kill the parasite *in vitro* at concentrations of less than one micromolar. These compounds are called *hits*. The next step is to show that the hits are orally available, are safe *in vitro* (when tested on mammalian cells), and kill the parasite *in vivo* (when it resides in the host). These compounds are called *leads*. The compounds are tested in a mouse model – either using the murine parasite *Plasmodium berghei* (Sanchez 2004) or the human parasite *P. falciparum* in a "severe combined immunodeficient" (SCID) mouse that is supplemented with human erythrocytes (Jimenez-Diaz 2009). The process from hits to leads typically takes one year, and the majority of the projects are not successful. Lead compounds are then further optimized, increasing plasma exposure and also increasing the safety margin where possible.

Once optimization is complete, the first *in vivo* safety data are obtained and further profiling is completed to judge whether the compound meets the criteria for a preclinical candidate. The first calculations of a predicted human effective dose become available at this stage, and combined with an early estimate of cost of goods, this gives an indication of whether the compound is likely to be part of a cure costing less than $1 for an adult. The process from leads to a preclinical candidate typically takes two more years, and, again, there is significant attrition.

Once optimized, the candidates enter preclinical development. This step typically takes 12 to 18 months and involves showing safety over 14 days in two mammalian species (typically rat and dog), as well as confirming safety in a variety of tests, especially cardiovascular studies. About 50% of projects successfully complete the preclinical stage, which cost around $2 million.

The Phase I clinical trial is the first opportunity to examine the safety of a potential new medicine in humans. A small group of closely monitored healthy human volunteers receive an initial dose, and, provided no unexpected adverse events occur, the dose is increased gradually and carefully up to the predicted human dose. If this can be safely achieved, volunteers can then be challenged with the blood-stage malaria parasite to give early data on pharmacological activity (McCarthy 2016; McCarthy 2013; McCarthy 2014a; McCarthy 2016; McCarthy 2014b; McCarthy 2011; Pasay 2016). These studies take about 6 to 12 months and typically cost between $1 million and $1.5 million. Malaria is one of very few diseases where it is possible to obtain critical pharmacodynamic parameters at such an early development stage. This greatly helps to limit the number of doses (and costs) to be tested later on in patients and in finding effective and safe dosages.

Malaria medicines will be marketed as combinations. The Phase II clinical trial program therefore consists of two parts. First, the potential medicines are tested as monotherapy in adult patients to confirm significant efficacy and safety of the new compound (Phase IIa). Then the combination of medicines (including the new experimental drug) is tested in healthy volunteers to check for any safety signal or strong drug–drug interactions. After this, compounds can be tested in combination in patients to establish the active dosages (Phase IIb). This development process necessitates an adaptive design because it would not be possible or ethical to study a wide range of combinatorial drug doses. Fortunately, the expectation is high: We are looking for medicines that are 95% effective, and so any dose combination that sees more than a couple of failures can be rapidly decided.

Two additional variables have to be considered in Phase II, increasing the challenge further. First, the study has to include pediatric patients as young as 1 year old. These patients are much more vulnerable, and such studies can only proceed once the safe dose of medicine has been established for adults. Second, the initial studies generally take place in immune-naive patients in Asia (these are patients whose host immune system does not bring significant additional help to clear the parasite). It is also important, given the variations in host (*e.g.*, immunity) and environmental (*e.g.*, strains, vector variation) factors to verify that the medicine is active in African patients.

Once these areas have been explored, the project can proceed to a definitive set of Phase III trials. The new medicine has to be compared head-to-head with the best treatment currently available, with at least 2,000 patients receiving the new medication. Data are obtained in as many countries as possible where the final product will be used. These trials last approximately three years in total, including the regulatory submissions and final report writing. These trials are designed to support registration, and the highest standard is with a stringent regulatory agency (such as the United States Food and Drug Administration, the European Medicines Agency, or the Japanese Pharmaceuticals and Medical Devices Agency).

Europe has a system, called Article 58, where the agency gives a view, in conjunction with the WHO, as to the suitability of the medicine for use outside of Europe. There are two advantages of this process: Once a positive opinion is given, then the medicine can be prequalified by the WHO; prequalification is the certification to allow the medicine to be purchased by UN agencies, and it is the stamp of approval for quality (http://apps.who/int/prequal/). The second advantage of article 58 is that the applying company is not required to market in Europe; under normal registration, if a company does not market in Europe, it would lose the approval.

Most important of all, the medicine then needs to be approved by the disease-endemic countries themselves. For the moment there is no centralized body, and so this has to be done on a country-by-country basis, a process that can take an additional two years. This process is being facilitated by

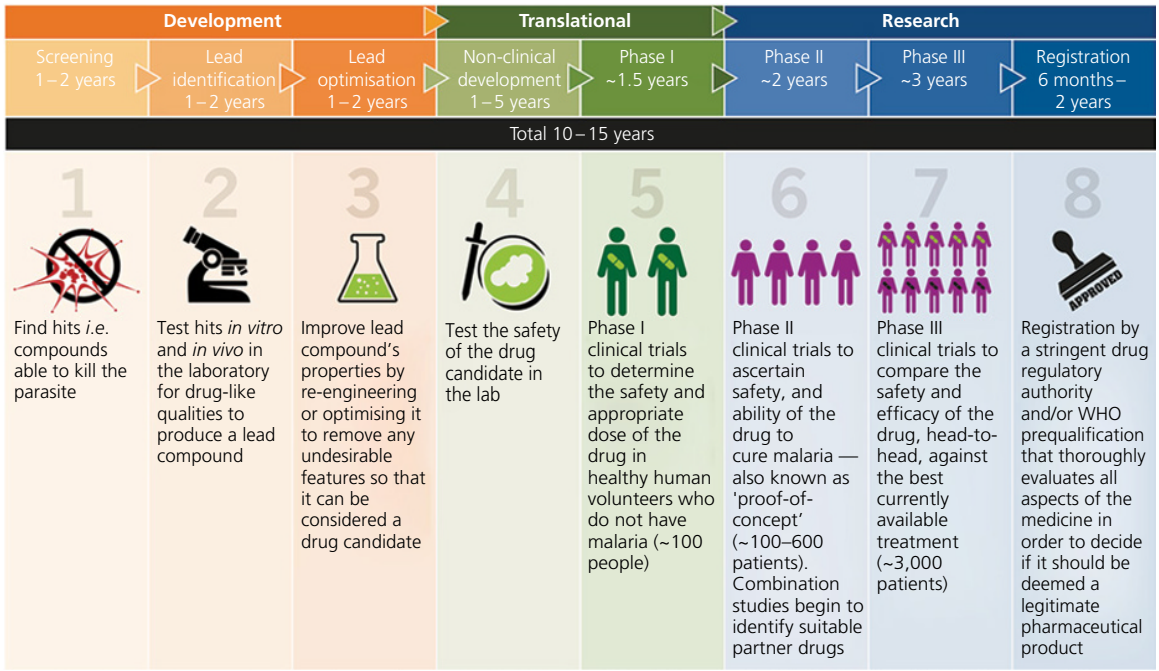

Figure 13.2 The research and development process for new antimalarial medicines, giving relative timelines for each phase. The times given are indicative, but historical timescales have often been significantly slower for a number of reasons, including irregular funding.

the African Medicines Regulatory Harmonisation (http://www.amrh.org/) and the East African community meeting on regional harmonization, which will accelerate the availability of medicines to the patients who urgently need them (Trade and Development Board 2012).

The entire drug-development process is shown in Figure 13.2.

Protecting new medicines from the emergence of resistance

As with any infectious disease, the threat of emergence of resistance is a constant challenge. The speed at which resistance could spread is always unpredictable, but its consequences are dramatic. To reduce the risk, two approaches are taken. Fixed-dose combination medicines act to slow the spread of resistance to the artemisinins and new classes of molecules. Also, screening candidate molecules at an early stage can help eliminate those that have a high potential for resistance development *in vitro* or that are less active against existing primary isolates (Ding 2012). When studying molecules *in vitro,* four parameters are important: how frequently a resistant strain appears; the physiological consequences, in terms of IC_{50} shift; the fitness cost to the parasite; and transmissibility. A standardized *in vitro* methodology to quantitatively assess these characteristics in *P. falciparum* has been proposed (Ding 2012). This should allow the selection of drug candidates that have a low potential for the development and transmission of resistance. Fitness costs and incompatibilities between traits for resistance against multiple drugs are not obvious. Only recently was it found that resistance against atovaquone results in a failure of the parasite to be transmitted by mosquitoes (Goodman 2016).

The number of new classes of molecules entering the pipeline has increased considerably following a renewed focus on whole-parasite screening, with more than seven new chemotypes entering

development (Wells 2015). This is a great step forward, but with the challenge of resistance, and the needs of new molecules against other lifecycle stages, this productivity needs to be continued for many years. It is important not to forget that a new candidate molecule has only a 50% chance of starting Phase I, and that only one in ten of the molecules entering Phase I in infectious disease will result in a product. With such low odds then, a large and diverse portfolio remains a priority.

Advances in drug development made in the 21st century

A number of major advances discussed below have greatly improved the efficiency of drug discovery. This is great progress, but it needs to be underlined that they are focused on the blood stages of *P. falciparum.* New techniques and models are still needed, specifically against *P. vivax* and the dormant liver stages, and for the measurement of the transmission from humans to humans.

High-throughput phenotypic screening

As described earlier in this chapter, many screens for new antimalarial compounds now directly test compounds against blood-stage parasites cultured *in vitro*. These are performed in 1,536-well plates in specialized centers such as the Novartis GNF site in San Diego, but they are also run by academic groups in 384-well systems, routinely using fluorescence-based assays (Guiguemde 2010). The availability of testing in an academic setting has led to new collaborative models for screening. Companies have been able to blind-test their compound sets in MMV-validated screening centers, and this means that questions about intellectual property can be discussed only once it is known whether there are any active compounds. The work is now moving beyond blood stages, and the race is on to produce good screening formats for the gametocyte or the dormant liver stages. The success of high-content screening in finding new chemotypes with activity against the erythrocytic stages suggests that this could also be applied to the other stages of the parasite lifecycle.

How fast compounds work: The parasite reduction ratio

Beyond a question of whether the molecule kills the parasite, one priority that emerges is whether the compound kills quickly (like artemisinin), with medium speed (similar to pyrimethamine), or has a slower onset (such as seen with atovaquone or azithromycin; White 2011). A new assay has been developed that allows the direct determination of *in vitro* killing rates for potential antimalarials (Sanz 2012). The new assay is based on limiting serial dilution of treated parasites and re-growth monitoring, which allows the direct *in vitro* measurement of the effect of antimalarial compounds on parasite viability. In addition, drug lag phase, which is the time required for a drug to achieve its maximal killing rate, can be precisely identified and timed. Although this assay was developed by a pharmaceutical company, GlaxoSmithKline, it has been made available to the entire community through agreements with Medicines for Malaria Venture and also through the Tres Cantos Open Laboratory Foundation (TCOLF; http://www.openlabfoundation.org/).

Pharmacokinetic and pharmacodynamic modeling

One of the most important issues is to be able to predict the human effective dose from the preclinical data. First, a variety of different approaches are being considered to predict the human pharmacokinetics. These include physiologically based pharmacokinetic (PBPK) predictions using metabolic clearance and other critical *in vitro* data; these can be refined and validated using *in vitro–in vivo* correlations from the preclinical pharmacokinetic studies. An additional approach is to use

allometry, *i.e.*, scaling the pharmacokinetics from preclinical species on the basis of body weight. Given the errors involved in such predictions, it is often highly desirable to rapidly move to Phase I and to actually measure the pharmacokinetics in humans. The early human pharmacokinetic predictions are important, however, because they can facilitate an early decision to terminate compounds that have no likelihood of ever achieving the necessary human profile, even taking error bars into consideration.

Modeling the *in vivo* parasite concentration response from the *P. falciparum* SCID mouse model is then performed, and a human dose prediction made using the predicted human pharmacokinetics along with the pharmacodynamic response from the preclinical model. This approach has already been demonstrated to predict the human parasite–concentration response for mefloquine given only the human pharmacokinetic and preclinical *in vivo* model data (McCarthy 2016). Our goal here is ultimately to validate these approaches and thus be able to integrate all of the preclinical efficacy data from the mouse models with the existing pharmacokinetic data (which will also include studies from rats and dogs). The key parameter to be determined here is how long a dose of the medicine can maintain plasma concentrations above the minimum parasiticidal concentration MPC (*i.e.*, the concentration above which there is maximum parasite killing).

Challenge models as proof of concept in humans

As mentioned above, there are some issues with the initial testing of compounds in human patients: particularly that the patients must be free from an anti-parasite immune response, which inevitably means they will be in low-transmission areas with seasonal malaria. An alternative is to test the medicines in volunteers who have been experimentally infected with drug-sensitive malaria. With the advent of PCR amplification, parasite levels as low as 10/mL to 100/mL can be detected – the limit with microscopy is often 3/μL. This means that the volunteers can be monitored for a couple of parasite lifecycles before they become symptomatic, and they can be treated with the standard of care before the point at which symptomatology would be expected to occur. The parasite clearance curves are similar to those seen in patient studies (although there are around 1,000-fold fewer parasites) (McCarthy 2013). The set of old and new compounds tested in this model is steadily expanding (McCarthy 2016; McCarthy 2013; McCarthy 2014a; McCarthy 2016; McCarthy 2014b; McCarthy 2011; Pasay 2016).

The need for new *in vitro* and *in vivo* models for *P. vivax*

Although *P. vivax* causes as much as 25% to 40% of the global malaria burden, particularly in South and Southeast Asia and Central and South America, it has a much smaller share of the research and development effort (Price 2007). Fortunately, medicines active against the *blood* stages of *P. falciparum* are usually also active clinically against *P. vivax*, although this assumption should always be adequately demonstrated. One remaining challenge is new safe and effective medicines to prevent *P. vivax* relapse (malERA Consultative Group on Drugs 2011). It is usually assumed that a relapse stems from the reactivation of dormant liver stages. Currently the only licensed medicine preventing relapse is primaquine, which has three challenges. First, it causes hemolysis in individuals with G6PD deficiency (which is common in malaria-endemic regions). Second, this means it is contraindicated in pregnant women because the fetus cannot be genotyped. Third, compliance is an issue, because a treatment course of 14 days is required, and there are no symptoms to encourage the patient to finish the course of treatment.

The first challenge for the development of new medicines to prevent *P. vivax* relapse is the availability of cellular screens for anti-relapse agents. A *P. vivax* liver-stage assay requires a supply of viable sporozoites. *P. vivax* continuous *in vitro* culture is currently not possible due to the selective

infection of reticulocytes, (immature red blood cells) by *P. vivax* (although reports of 1-month culture suggest a breakthrough in this area is possible). Next, stable and infectable hepatocyte lines are needed; primary human liver cells vary in quality and rapidly de-differentiate in culture. Infection rates remain low for *P. falciparum* (typically <0.1%), suggesting that a critical co-factor or co-receptor is yet to be identified. Higher infectivity has been seen with *P. vivax* and the cell line HepG2, but this cell line has significant metabolic differences from primary hepatocytes.

With no cell assay for *P. vivax* relapse, anti-relapse drug discovery has been focused on a surrogate assay using *P. cynomolgi* infection of rhesus monkey primary hepatocytes (Dembele 2011). The advantage here is that sporozoites are much easier to generate and also that primary hepatocytes are more widely available and metabolically active, allowing primaquine to be used as a control. Even so, this assay represents a huge undertaking, and the throughput is not large (between 100 and 200 compounds per year). For high-throughput screening, the only alternative for now is to screen against the liver stages of *Plasmodium yoelii* or *P. berghei,* which do not appear to have a dormant form of parasite, but this at least helps to prioritize anti-plasmodial compounds with hepatic stage activity for further anti-relapse testing (Delves 2012).

For the *in vivo* assay, the situation is somewhat more promising, because *Plasmodium cynomolgi* can be tested directly in primates. This parasite relapses in a regular time frame (around 22 days), and thus new compounds can be dosed once the blood stages have been cleared (using chloroquine) and the animal parasitemia monitored for a relapse. However, these studies are expensive, (around $250,000 for a dose-response). Mice have been suggested as an alternative pharmacological model. SCID mice with human erythrocytes are used to study the erythrocyte stages of *P. falciparum* (Angulo-Barturen 2008; Jimenez-Diaz 2009), and SCID mice with human livers are also being explored. These models offer a more ethically acceptable alternative than primates, but it remains to be seen whether they are more cost effective, and also the current inoculation volumes (a million sporozoites per mouse) means that there is still much to be done to increase their pathological relevance.

Clinically, the challenge is to distinguish relapses caused by reactivation of dormant stages from reinfection. This is in contrast to the situation with *P. falciparum*, where recrudescence and relapse are distinguishable by PCR (Chen 2007; Collins 2007; Imwong 2007). The only way to be certain that new parasitemia results from relapse is to work with subjects who return to an area free from any risk of reinfection, and who remain there for a follow-up period that can be as long as 12 months (Baird and Rieckmann 2003). In this trial design, parasitemic subjects receive a fast acting co-therapy to kill blood-stage parasites to eliminate recrudescence, and the test medicine is given as a co-therapy once it is established that there are no clinically significant drug interactions in patients.

An alternative approach is being followed by GlaxoSmithKline for the new 8-aminoquinoline, tafenoquine (ClinicalTrials.gov Identifier: NCT01376167). The protocol compares tafenoquine–chloroquine combinations with chloroquine alone (chloroquine is used as the schizonticide, because it is still active against blood stages of *P. vivax* in many places). This protocol does not distinguish relapse from reinfection (the assumption is that the two are equally bad for the patient), and it implicitly assumes that because few complete 14 days of primaquine treatment, it cannot be assumed to be the standard of care.

A drug that targets the hypnozoite stage of *P. vivax* is undoubtedly the greatest challenge facing the malaria elimination agenda. To successfully meet this challenge, it is clear that a number of new methodologies and *in vitro* models will need to be developed for *P. vivax*. Testing the existing portfolio of blood-stage molecules in these assays is the most likely way that new low-hanging fruit will be found in this area.

The global pipeline of new medicines for treating malaria

Next-generation endoperoxides

MMV established a project with the University of Nebraska, the Swiss Tropical and Public Health Institute, and Monash University in 2000 to develop synthetic endoperoxides to replace artemisinin. A first-generation compound, OZ277/Rbx11160, was launched in combination with piperaquine under the combination name Synriam by Ranbaxy in 2012 in India. The Phase III data have been published (Toure 2016). A second-generation molecule, with far superior pharmacokinetics, OZ439 (Charman 2011; Moehrle 2013), has completed Phase II safety studies (Phyo 2015), and efficacy studies in combination with ferroquine were started in 2015. Studies with OZ439 in both healthy volunteers (McCarthy 2016) and patients (Phyo 2015) confirm a significant plasma exposure after 6 days, suggesting it might be used as part of a single-dose therapy for uncomplicated malaria (Kortagere 2010). In patients, OZ439 drives the reduction of parasites at about the same speed as artesunate. Preliminary evidence suggests that parasites that carry mutations that make them partially resistant to artemisinins still respond fully to OZ439 (Phyo 2015). In transmission-blocking studies, OZ439 is active at preventing the formation of the oocyst as measured in membrane (mosquito) feeding assays, suggesting a role in transmission blocking.

The next step is to identify a suitable combination partner that will give long-term protection and support a single-dose cure. The current candidates include the 4-aminoquinolines naphthoquine and ferroquine, as well as the aminoalcohol mefloquine (being tested), but the challenge is to find one that would be suitable when given as a single dose (Duparc 2012). It may be that none are suitable and that the single-dose cure will have to await the discovery and development of new partner medicines.

The other key question is whether such medicines would work against the artemisinin-insensitive strains of the parasite. In 2009, parasites with delayed parasite clearance were reported in the Thailand–Cambodia border region; parasites had a clearance half-life of around 6 hours compared with approximately 4 hours in Thailand and 2 hours in Laos (Noedl 2008). Reports suggest a similar but slightly smaller shift in parasite clearance time in western Thailand, where over 10 years the drug response half-lives have increased from 2.6 to 3.7 hours (Noedl 2008; Phyo 2012). These increases are putting additional pressure on the partner drugs, which must face higher numbers of parasites as monotherapy. Studies have shown that although both DHA–piperaquine and artesunate–mefloquine clear the parasite more slowly, in the same year they are still capable high efficacy cures at day 28 (Leang 2012). This suggests that the interpretation of these results is complicated by the resistance to partner drugs, and one interpretation is that it is resistance to the partner drug that is causing the clinical failure. In any case, it is not easy to identify artemisinin-resistant patients to test new medicines: they are mainly focused in one or two districts of Cambodia, with only a handful patients identified each month, and these individuals are being adequately treated by the WHO-led containment programs and are thus not available for testing new classes of medicines.

Cell biological approaches to confirm the activity of OZ439 against artemisinin-insensitive malaria have also been difficult, because *in vivo* responses were often difficult to reproduce *in vitro*. A breakthrough has been achieved with the development of the *in vitro* ring-stage survival assay (RSA) (Witkowski 2013).

Beyond the endoperoxides: Overview of the pipeline

New drugs with completely new mechanisms of action would be expected to have fewer problems demonstrating activity against artemisinin-insensitive malaria. The frontrunner is KAE609, a spiroindolone developed by Novartis at their Institute for Tropical Diseases in Singapore (see below). Currently there are seven other molecules in translational sciences (a name used for the activities

between the start of regulatory preclinical safety studies and human proof of concept). Several other molecules have advanced to this stage, but these are not progressing in development (Wells 2015).

KAE609 (Cipargamin)

KAE609 is a novel synthetic spiroindolone developed by the Novartis Institute for Tropical Diseases (NITD) in Singapore in collaboration with the Swiss Tropical and Public Health Institute (Swiss TPH), the Dutch Biomedical Primate Research Center, and the Genomics Institute of the Novartis Research Foundation (GNF) in San Diego. It was the first compound to be developed using parasite-focused screening (Rottmann 2010) and started human proof of concept in 2012, just under 5 years after the start of screening. It inhibits the P-type sodium transporter ATPase 4 (*Pf*ATP4), which had previously been characterized but not prioritized as a target for antimalarial compounds (Krishna 2001). Inhibition of this parasitic ion channel may also cause a change in the flexibility and shape of the infected erythrocyte, which increases splenic clearance. Splenic microcirculatory beds filter out altered erythrocytes, meaning that the spleen can innately clear subpopulations of infected or uninfected erythrocytes modified during falciparum malaria (Buffet 2011). KAE609 is one of only a handful of molecules capable of completely curing a *P. berghei* model of blood-stage malaria. In humans the compound is well tolerated, with a half-life consistent with daily dosing, and it is potentially part of a single-dose cure (Rottmann 2010; Yeung 2010; Huskey 2016). The exciting results from the first tests in patients show it kills parasites even faster than artesunate (White 2014). In addition, it kills *P. falciparum* gametocytes and is active in a standard membrane feeding assay, suggesting it has the potential to be transmission blocking.

KAF156 (GNF156)

KAF156 is an imidazolo-piperazine identified by the Genomics Institute of the Novartis Research Foundation (GNF) in San Diego, again in collaboration with MMV. It exhibits *in vitro* potency against blood stages, liver schizonts (but not the hypnozoites), and gametocyte stages, and it was shown to be highly active in murine models (Meister 2011; Kuhen 2014). The series acts via a novel mechanism, involving a previously unannotated gene now called *P. falciparum* cyclic amine resistance locus *(Pfcarl).* The compound is well tolerated in preclinical safety studies, and it has now safely completed Phase I studies and completed a human proof-of-concept study in patients with *P. falciparum* or *P. vivax* in Thailand in early 2013.

Actelion antimalarial ACT451840

The Swiss biotechnology company Actelion, in collaboration with Swiss TPH, has identified an antimalarial compound from a parasite screen of a highly focused set of molecules. This compound, which has *in vitro* parasite-reduction rates similar to those of chloroquine, has been successfully taken through preclinical development and was tested in human volunteers as part of a Phase I study (Bruderer 2014). The molecule has been found to effectively clear parasites in infected volunteers (Krause 2016). Actelion was looking to out-licence this molecule as a result of internal refocusing of resources.

P218

Dihydrofolate reductase (DHFR) inhibitors such as pyrimethamine have been widely used for the treatment of malaria, although their clinical efficacy has been compromised in some regions by resistance via mutations in the enzyme. P218 is a next-generation inhibitor of DHFR with a high affinity for wild-type and clinically relevant resistant strains, and it has a good pharmacokinetic profile (Yuthavong 2012). P218 is a selective, highly efficacious, and orally available antimalarial drug candidate. The initial safety testing indicated a good safety margin between the toxicity in animals and the predicted effective human dose. The project has been led by the Thai

BIOTEC group in collaboration with Monash University, the London School of Hygiene and Tropical Medicine, and MMV; it has largely finished preclinical development.

DSM265

The *P. falciparum* enzyme dihydroorotate dehydrogenase (*Pf*DHODH) is known to be essential for the survival of the parasite. The potent and selective triazolopyrimidine-based inhibitor DSM1 was identified via high-throughput screening using an enzyme-based assay at the University of Texas, and the three-dimensional structure of the enzyme–inhibitor complex was resolved (Phillips and Rathod 2010). The subsequent lead optimization program funded by the U.S. National Institutes of Health in collaboration with Monash University, the University of Washington, GSK, and MMV led to the identification of an improved version, DSM265, which has exhibited good activity against *P. falciparum* both *in vitro* and *in vivo* and has an excellent safety profile in exploratory toxicology studies. It will be the first antimalarial chemotherapy to target DHODH. The molecule was tested in the human challenge model(McCarthy 2014a), and its full preclinical package has been published (Desai 2010; Phillips 2015; Phillips and Rathod 2010; Skerlj 2011).

PA21A092

PA21A092 is a potent and selective pyrazole that emerged from an *in silico* screening activity at DrexelMed University. Further optimization, in collaboration with the University of Washington, Novartis, and MMV, led to the candidate. It has good activity against *P. falciparum in vitro* and impressive *in vivo* activity in a *P. falciparum* laboratory model. The compound is undergoing preclinical GLP safety studies in preparation for the initiation of Phase I trials.

MMV390048

MMV390048 is a compound from the aminopyridine class that has the potential to become part of a single-dose cure for all strains of malaria. In addition, this compound has potential transmission-blocking properties. It was identified as a result of a collaboration between MMV and the Drug Discovery and Development Centre (H3-D) at the University of Cape Town, South Africa. It came from whole-parasite screening of a diversity collection from Biofocus. It has been found to target *Pf*PI4 kinase (Ghidelli-Disse 2014). The compound has been tested in Phase I trials and the human challenge model. Completion of Phase I/Ib studies with new formulation is scheduled for 2016.

Overview of the global malaria portfolio

The pipeline of new molecules targeting malaria remains rich, with at least seven new compound families having been discovered since 2009. Table 13.2 outlines the new medicines in development and their targets.

This strong portfolio reflects the commitment of the field as a whole. However, because the probability of a Phase I molecule making it all the way through the pipeline to launch has been estimated at just 20%, and because two molecules are needed for each combination medicine, the pressure on identifying preclinical candidates and moving them through the pipeline is immense. Figure 13.3 shows the global portfolio of antimalarial medicines currently under development organized by development stage.

Medicines in the broader context of malaria eradication

There are two areas where medicines can be used to protect patients. First, there are vulnerable populations, such as small children and expectant mothers. Second, as countries become malaria free, their populations will lose their immune protection, and prophylaxis will be required for them

Table 13.2 New malaria medicines in development and their targets.

Active ingredients	Partnership	Phase	Comments
DSM-265	University of Texas USA; Monash University, Australia; University of Washington, USA; MMV	Phase II	A dihydroorotate dehydrogenase (DHODH) inhibitor with good activity against the *P. falciparum* orthologue both *in vitro* and *in vivo* and an excellent safety profile in exploratory toxicology studies. It would be the first antimalarial chemotherapy to target *Pf*DHODH, which is essential for parasite survival. The compound is expected to enter Phase II trials in 2016.
P218 DHFR	BIOTEC; Monash University, Australia; London School of Hygiene and Tropical Medicine, London, UK; MMV	Preclinical	A dihydrofolate reductase inhibitor that binds with high affinity to the wild-type and resistant dihydrofolate reductase (DHFR) enzymes. Drugs that inhibit the folate pathway, such as sulfadoxine–pyrimethamine, have been widely used for the treatment of *P. falciparum,* but their efficacy is declining due to the emergence of drug resistance via mutations in DHFR. The aim of this project is to develop P218 as a curative antifolate agent potent against these resistant strains. Initial safety testing indicated that P218 has a good safety margin between the toxicity in animals and the predicted effective human dose.
PA21A092	Drexel University College of Medicine, PA, USA; University of Washington, USA; Monash University, Australia; GNF, Novartis; MMV	Preclinical	A potent and selective pyrazole targeting *Pf*ATP4 with good activity against *P. falciparum in vitro* and impressive *in vivo* activity in a *P. falciparum* laboratory model. The compound is undergoing Good Laboratory Practice toxicology and safety studies in preparation for the initiation of Phase I trials.
MMV390048	University of Cape Town, South Africa; Swiss Tropical and Public Health Institute, Switzerland; Monash University, Australia; MMV	Phase I	A novel antimalarial compound belonging to the aminopyridine class that was selected for preclinical development in July 2012. It targets *Pf*PI4K and shows potent activity against multiple stages of the malaria parasite lifecycle, with potential for transmission blocking. The compound is completing Phase I and human volunteer challenge model trials.
NPC-1161-B	University of Mississippi, USA	Preclinical	A novel 8-aminoquinoline antimalarial agent that has been shown to inhibit the production of sporozoites and to block relapse. It is reported to have less hemolytic toxicity in G6PD-deficient blood.
SAR116242 / PA 1103	Palumed, Castanet-Tolosan, France	Preclinical	A fusion compound containing a trioxane ring and a 4-aminoquinoline group. It entered preclinical development in 2007 but has not progressed into human studies.
KAF156	Genomics Institute of the Novartis Research Foundation, CA, USA; Swiss Tropical and Public Health Institute, Switzerland; MMV	Phase II	An imidazolopiperazine with novel mechanism of action active against blood, liver, and gametocyte stages of *Plasmodium* and active in murine models. It has completed Phase IIa studies in patients with *P. falciparum* or *P. vivax* infection.

(*Continued*)

Table 13.2 (Continued)

Active ingredients	Partnership	Phase	Comments
CDRI 97-78	CDRI, Lucknow, India; Ipca, Mumbai, India	Phase I	A water-soluble synthetic derivative active in both rodent and monkey malaria models. Regulatory toxicity and pharmacology studies have been completed. Phase I safety trials have been conducted in 50 volunteers, and phase I single-dose pharmacokinetics trials have been completed in 16 volunteers.
AQ-13	Immtech, NY, USA	Phase IIa	AQ-13 is a 4-aminoquinoline active against chloroquine-resistant *P. falciparum* infection. A phase I study with 126 individuals was successfully completed in 2005, but no further development was reported. The compound has no significant advantages over other 4-aminoquinolines.
OZ439	University of Nebraska Medical Center, USA; Monash University, VIC, Australia; Swiss Tropical and Public Health Institute, Switzerland; MMV	Phase IIa	Next-generation synthetic peroxide. Phase I showed OZ439 was safe at doses up to 1,600 mg as a single dose, and it gives plasma concentrations with anti-parasite activity up to 72 hours. A Phase IIa study in *P. falciparum* and *P. vivax* patients is complete.
KAE609	Novartis Institute for Tropical Diseases, Singapore; Swiss Tropical and Public Health Institute, Switzerland; MMV	Phase IIa	Spiroindolone suppresses parasite growth working through PfATP4. NITD609 has the pharmacokinetic properties compatible with at least once-daily oral dosing for the potential treatment of falciparum and vivax malaria. It has completed Phase IIa studies (short-duration monotherapy proof-of-concept in patients), and a controlled human induced blood–stage malaria challenge study is ongoing in healthy volunteers.
SSR-97193 (Ferroquine), artesunate	Sanofi, Paris, France	Phase IIa	A 4-aminoquinoline active *in vitro* against multidrug-resistant *P. falciparum*. Phase II studies showed that it is active in combination with artesunate in African adults and children. Project was put on hold, presumably because of insufficient product differentiation.
Fosmidomycin piperaquine	Jomaa Pharma GmbH, Hamburg, Germany	Phase IIa	A proof-of-concept study of fosmidomycin in combination with piperaquine was planned to take place in Thailand. This will be followed by Phase II studies in children and toddlers in sub-Saharan Africa.
Methylene blue amodiaquine	University of Heidelberg, Germany	Phase IIa	Methylene blue was first proposed by Ehrlich as a potential antimalarial molecule. It is being tested in Africa in combination with amodiaquine. It is a registered drug in many countries, which facilitates its development.
SAR97276	Sanofi , Paris, France	Phase IIa	SAR97276 is a bis-thiazolium compound that targets phospholipid metabolism of *P. falciparum*. It is planned to be given by injection for severe malaria but is currently in Phase II studies to determine its efficacy against uncomplicated malaria.

Artemisone (semi-synthetic artemisinin derivative)	Hong Kong University of Science and Technology	Phase IIa	Currently in preclinical studies, a new artemisinin derivative, artemisone, is being discussed as an option for treating artemisinin-resistant malaria, although clinical trials have not started due to lack of patient populations and stable material.
Azithromycin 250 mg, chloroquine 155 mg	Pfizer; London School of Hygiene and Tropical Medicine, UK; MMV	Phase IIb/III	Azithromycin is a macrolide requiring 2,000 mg/day to be effective as monotherapy but shows clinical synergy with chloroquine even where chloroquine resistance is as high as 50%. Entered Phase III for intermittent preventive treatment of *P. falciparum* malaria in pregnancy in October 2010. The primary outcome is a reduction in the number of subjects with suboptimal pregnancy outcome, and results should be available in 2013.
Tafenoquine	GlaxoSmithKline, UK; MMV	Phase IIb/III	Tafenoquine is MMV's lead investigational medicine for the treatment of *P. vivax* (relapsing) malaria. It is an 8-aminoquinoline, of the same family as primaquine (current standard of care for preventing relapse), with demonstrated activity against the dormant liver form of *P. vivax in vitro* and in patients. As for primaquine, patient testing for G6PD deficiency is required. Phase IIb study data confirmed that a single 300-mg dose resulted in 89.2% relapse free at 6 months. A large Phase III study started in 2014, with expected completion in 2016.
Pyramax paediatric	University of Iowa, USA; Shin Poong Pharmaceuticals, South Korea; MMV	Post-approval	The tablet formulation was granted a positive scientific opinion from the European Medicines Agency under Article 58 in February 2012. A child-friendly granule formulation has also been developed specifically for use in pediatric patients from 3 months (5 kg body weight). A randomized, controlled, multicenter Phase III trial was performed to assess the efficacy, tolerability, and safety of the Pyramax Paediatric granule formulation compared to artemether–lumefantrine treatment (crushed tablet) in more than 500 children up to 12 years of age with *P. falciparum* malaria. The trial achieved its primary endpoints, demonstrating the high level of efficacy of Pyramax in this patient population. The drug was well tolerated and the granule formulation was shown to be easy to administer. Positive opinion from EMA (Article 58), new label (tablets), and approval (granules) occurred in 2015. It is cross-listed on the WHO prequalification medicines site.
Eurartesim paediatric	Sigma-Tau, Gaithersburg, MD, USA; MMV	Post-approval	The European Medicines Agency (EMA) has granted regulatory approval for Eurartesim, dihydroartemisinin–piperaquine (DHA/PQP), developed in partnership by Sigma-Tau and MMV for the treatment of uncomplicated *P. falciparum* malaria. The WHO Prequalification Team has added Eurartesim to its list of prequalified medicines.

(*Continued*)

Table 13.2 (Continued)

Active ingredients	Partnership	Phase	Comments
Synriam	Ranbaxy, Gurgaon, India; MMV	Phase IIb/III	Rbx11160/OZ277 150 mg, piperaquine phosphate 750 mg (Arterolane maleate + piperaquine phosphate) is a first-generation synthetic peroxide (trioxolane), given in combination as one tablet per day for three days. Arterolane as a monotherapy drug showed lower efficacy than artesunate with 7 days of dosing. A Phase III trial in India, Bangladesh, and Thailand has been completed, and this has been used as the basis for approval in India.
Trimethoprim/ sulfamethoxazole (Co-trimoxazole)	Studies by Institute of Tropical Medicine, Antwerp, in Zambia and by UCSF in Uganda	Phase III for malaria	An antibacterial with activity against malaria. Early data in children showed similar efficacy to amodiaquine artesunate. A Phase III study comparing against sulfadoxine–pyrimethamine and dihydroartemisinin-piperaquine was expected to be completed in July 2014. A Phase III study with 1,714 subjects in Zambia is testing its effectiveness as prophylaxis to prevent malaria in pregnancy.
ARCO Naphthoquine/ artemisinin	Kunming, China	Phase IIb/III	A combination of naphthoquine and artemisinin that is given in a single encounter, over multiple pills, to reportedly cure malaria. No approval from a stringent regulatory authority.
ArtiMist	Suda Ltd., India	Phase III	ArtiMist treatment is a sublingual dose of artemether for severe malaria (administered under the tongue). It has completed clinical efficacy evaluation in a Phase III trial, and the regulatory dossier is being prepared.

Research	Translational			Development		
Lead optimisation	Preclinical	Phase I	Phase IIa	Phase IIb/III	Registration	Phase IV
Pf DHODH inhrs.	P218	MMV390048	OZ439/FQ	Tafenoquine	Rectal ART	Artemether-lumefantrine Dispersible
Tetraoxanes	DDD107498	ACT-451840	KAE609	DHA-PPQ Paediatric	Arterolane/ PPQ	ART for injection
Heterocycles	PA21A092	CDRI 97/78	KAF156	Co-trimoxazole		DHA-PPQ
Pf NMT inhrs.	MMV253 (AZ13721412)	SJ557733	DSM265	Artemisinin Naphthoquine		Pyronaridine-ART
Pantothenamides	GSK130 (GSK3212030A)		Sevuparin	Artemether sub-lingual spray		Pyronaridine-ART Paediatric
Pf NDH2 inhrs.	DSM421					ART-amodiaquine
Imidazolidinediones	AN762					ART-mefloquine
dUTPase inhrs.	JPC2997					SPAQ (Sulfadoxine-pyrimethamine + amodiaquine)

Figure 13.3 The global malaria portfolio at the end of 2012. inhrs.: inhibitors.

in the same way as it is currently needed for Western travellers. In both cases, the individuals receiving these medicines will not have clinical disease, and consequently, the risk-to-benefit ratio will have to be extremely low (fewer than 1:10,000 serious adverse events). In addition, the medicines used for chemoprevention strategies must not be the same as those used for treatment so as to reduce the risk for development of resistance. This puts further pressure on the need for discovery of as many new types of medicine as possible.

Chemoprevention in vulnerable populations

The use of drugs to prevent malaria in endemic countries is currently focused on the vulnerable populations of children and pregnant women. WHO has recommended a policy of SMC as a malaria-control strategy in children younger than 5 years for the Sahel subregion of Africa (WHO 2015a). This means giving full treatment courses of amodiaquine plus sulfadoxine–pyrimethamine every month during the season, to prevent symptomatic malaria. In children aged between 3 and 59 months there was a decrease in malaria incidence of 80% (WHO 2012). This drug combination has an extremely low cost, with a potential of 50 cents per child per year (Dicko 2011; Konate 2011). This sets an interesting threshold for both the price and efficacy of any vaccine, and it needs to be taken into consideration with the recent news on RTS,S, where the efficacy was closer to 30%, and presumably the price will be higher (The RTS,S Clinical Trials Partnership 2012) cited at $15 per dose (Seo 2014). All prevention measures will require an increased investment in pharmacovigilance, to enable proper evaluation of this approach. A similar policy known as intermittent preventive therapy during infancy (IPTi) involves administering a full therapeutic course of sulfadoxine–pyrimethamine to infants at risk for malaria alongside the routine vaccination schedule – usually at 10 weeks, 14 weeks, and 9 months of age.

Malaria in pregnancy is the most common yet preventable cause of maternal and perinatal morbidity and mortality in sub-Saharan Africa. Around 125 million pregnancies are at risk for malaria every year, and up to 200,000 babies die as a result (Dellicour 2010). Currently, intermittent preventive therapy in pregnant women (IPTp) (combined with the use of insecticide-treated nets

[ITNs]) is the strategy employed to protect women and their unborn children in areas of high malaria transmission. IPTp is the administration of a curative dose of an antimalarial drug during the second and third trimester at antenatal clinics, regardless of whether the expectant mother has malaria symptoms (WHO 2007). Treatment must be with a drug with a long half-life, on at least two occasions during the pregnancy, so that any existing malaria infection is treated, and the woman is protected from future infection or reinfection for some time following her visit to the clinic. In total, 35 of 45 sub-Saharan African countries had adopted IPTp with sulfadoxine–pyrimethamine as a national policy by the end of 2010 (WHO 2015a).

However, since this strategy was first recommended in 1998, resistance to sulfadoxine–pyrimethamine has increased considerably and now threatens the efficacy and effectiveness of IPTp with this drug. In 2007, a WHO Technical Expert Group concluded that the evidence available suggested that the benefits of sulfadoxine–pyrimethamine IPTp, given two or three times during pregnancy to women residing in areas of stable malaria transmission, still outweighed the risks. A study conducted in 2007 showed that this policy reduced the risk of low birth weight in first and second pregnancies.

With resistance to sulfadoxine–pyrimethamine increasing, new antimalarial regimens for IPTp are urgently needed. Owing to the difficulties of proving the safety of a drug in pregnancy, it has been difficult to find an alternative. Therefore, WHO continues to recommend the use of sulfadoxine–pyrimethamine IPTp for pregnant women at risk for *P. falciparum* in sub-Saharan Africa (WHO 2015a). New medicines will not be used in this vulnerable population until they have been shown to be safe in pregnancy through registries of inadvertent use. Mefloquine, azithromycin dihydrate, chloroquine phosphate, and piperaquine are all being studied for IPTp in falciparum malaria (Adam 2012; Briand 2009; Chico 2008).

Vaccines for the chemoprevention of malaria

In a viral disease, the role of chemoprevention would be taken by a vaccine. Development of an effective vaccine against malaria has proved difficult, and the parasite has evolved sophisticated systems for living alongside the human immune system. There is currently one (EMA-) approved malaria vaccine, RTS,S, or Mosquirix, and more than 20 candidates in research and development. RTS,S/AS01 was developed through a partnership between GlaxoSmithKline Biologicals and the PATH Malaria Vaccine Initiative (MVI).

A Phase III trial of RTS,S in more than 15,000 children in Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and the United Republic of Tanzania began in May 2009. The target population was children aged 6 to 14 weeks, for co-administration with other vaccines; however, the trial was also studying children aged 5 to 17 months at first dose who received only the RTS,S vaccine. The first interim report, published in October 2011, showed a promising efficacy of 55% reduction in frequency of malaria episodes during the 12 months of follow-up in children 5 to 17 months of age at first immunization (Agnandji 2011).

The initial results from Phase III were somewhat less encouraging, with vaccine efficacy in children 6 to 12 weeks of age at 30.1% (RTS,S Clinical Trials Partnership 2012). Additional analyses (Agnandji 2015; RTS,S Clinical Trials Partnership 2015) have shown moreover that the vaccine's efficacy wanes over time (Olotu 2013), and studies are ongoing to decide on how the vaccine can be deployed in a cost-effective manner (Penny 2015).

RTS,S is a *P. falciparum* vaccine, with no protection expected against *P. vivax* malaria. Therefore, despite the ongoing search for a vaccine, drugs will certainly have a key role to play in the long-term aim of global malaria eradication.

Conclusion

The landscape of malaria drug discovery has improved significantly over the last few years. Several new chemotypes have entered preclinical evaluation, and the most advanced of these are now in Phase II evaluation and are showing great promise. Set against this positive signal is the enormousness of the task ahead. Calls for malaria elimination mean that new types of medicines are needed, and if we are to succeed, several successful medicines will be needed in each class. Because only a few of the candidates that enter clinical trials survive a steady pipeline of new molecules will be needed for at least the next decade or so.

The development of open partnerships and effective communication channels will be crucial to ensure that the challenges to malaria eradication can be efficiently met and overcome. One bright light here is the increase in the amount of open-source drug discovery taking place within the field of malaria and the openness of the pharmaceutical companies to share not only their compounds but also their expertise to allow the progression of molecules.

Despite many new classes of antimalarials being identified and progressing along the pipeline, the malaria parasite and its vector will continue to evolve, ensuring that the threat of parasite resistance remains high on the research agenda. Continued funding for the development and also the deployment of new medicines is essential if the victories over the parasite achieved in the last few years are to be extended further.

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CHAPTER 14
Antimalarial drug resistance

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Background

Treatment failure and drug resistance

Antimalarial drug resistance has been defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (World Health Organization 1967). Forms of antimalarial drug resistance include cross-resistance, which can occur among compounds from similar chemical classes or those with similar mechanisms of action. Multidrug resistance is when a parasite strain demonstrates drug resistance to two or more compounds from different chemical classes and mechanisms of action.

A closely related yet distinct concept from antimalarial drug resistance is treatment failure. Treatment failure is a broader outcome and is characterized by the failure to clear parasitemia or the failure to resolve clinical symptoms after the administration of antimalarial therapy. Compared to antimalarial drug resistance, it does not necessitate the demonstration of adequate drug absorption and appropriate drug levels. Treatment failure can also depend on age, particularly in high-transmission settings where age serves as a marker of acquired immunity to malaria (Baird 1995; White 2002). Such immunity is important in directly clearing existing parasitemia and in preventing the accumulation of high parasite densities in the first instance (Doolan 2009). Thus, treatment failure may be conceived as reflecting the combination of host immunity and pharmacokinetics, in addition to parasite resistance to drugs. As such, it represents the interaction of many potential causes ranging from immunodeficiency and malnutrition to the timing of drug dosing or variations in enzymes responsible for drug metabolism.

The two concepts are complementary, and both provide useful information for different aims. Widespread treatment failure regardless of cause is a control program emergency, whereas treatment success in the presence of defined antimalarial drug resistance is likely fleeting and needs to be closely monitored.

Current status

The current state of affairs for drug resistance is sobering (World Health Organization 2010). Decreasing efficacies of antimalarial drugs have compromised the global malaria control strategy. Adopted in 1992 at a ministerial conference in Amsterdam, the strategy aims to reduce morbidity and mortality by providing early diagnosis and effective treatment (World Health Organization 1993). All known drugs, including artemisinin derivatives, have established clinical resistance

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Table 14.1 Time difference between the date of introduction and the first report of clinical resistance for selected drugs

Antimalarial Drug	First Introduced	First Reported Resistance	Time (years)	References
Quinine	1632	1910	278	Peters 1987
Chloroquine	1945	1957	12	Wernsdorfer 1991
Proguanil	1948	1949	1	Peters 1987 Björkman and Phillips-Howard 1990
Amodiaquine	1951	1961	10	Young 1961
Sulfadoxine–pyremethamine	1967	1967	0	Wernsdorfer 1991 Björkman and Phillips-Howard 1990
Mefloquine	1974	1982	8	Boudreau 1982
Piperaquine	1978	1985	7	Basco and Ringwald 2003
Halofantrine	1984	1990	6	Horton 1988
Atovaquone	1996	1996	0	Looareesuwan 1996

Data from Wongsrichanalai 2002.

(Table 14.1). Furthermore, the parasite develops resistance to antimalarial drugs faster than new compounds can be produced. *Plasmodium falciparum* resistance to chloroquine, the mainstay of malaria treatment for decades, is nearly universal and mirrors the distribution of the disease itself (Ringwald 2005). Lastly, declines in sulfadoxine–pyrimethamine cure rates in sub-Saharan Africa and mefloquine cure rates in Southeast Asia threaten to leave governments without a safe, effective, and inexpensive therapy (Ringwald 2005). *Plasmodium vivax* is now chloroquine-resistant across most of Brazil and Oceania, particularly Indonesia and Papua New Guinea, and it appears to be spreading, with reports from different countries in Southeast Asia and South America (Price 2012).

The current first-line therapy for *P. falciparum* (and chloroquine-resistant *P. vivax*) worldwide are the highly effective artemisinin combination therapies (ACTs). Combination treatments, particularly artemisinin-based therapies, are touted as a means to both provide effective treatment and prevent drug resistance (White 1996). In spite of the advantages of artemisinin from a drug-resistance perspective, rapid parasite load reduction, short half-life, and partial gametocytocidal activity, reduced sensitivity in the form of prolonged clearance times along the Thailand–Cambodia and Thailand–Myanmar borders suggests emerging drug resistance (Dondorp 2009). The spread of artemisinin-resistant falciparum malaria would be a public health disaster, and intensive containment efforts have therefore been initiated (World Health Organization 2011). Finally, primaquine, the only fully gametocytocidal and hypnozoitocidal drug in use, is suspected to have reduced sensitivity in parts of the world, but no assay exists to monitor or validate this (Baird and Hoffman 2004).

Public health burden

The public health costs of drug resistance are enormous. Drug resistance increases morbidity, mortality, parasite transmission, and the cost of malaria control. Increased drug resistance has been associated with increases in hospital admissions, anemia, low birth weight, and mortality (Phillips and Phillips-Howard 1996; Yeung 2004; Laxminarayan 2004). In an analysis of global

malaria deaths from 1980 to 2010, Murray and colleagues report that the major driver of increased mortality in their models was drug resistance against the first-line antimalarial treatment (Murray 2012).

Treatment failure is a strong risk factor for gametocytemia, and indeed gametocytemia rates were used as a marker for increasing drug resistance in South Africa (Barnes 2008). The clearest indicator of the transmission-enhancing effect of drug resistance is reports of rapid reductions in malaria incidence in Zanzibar, Thailand, India, and many other countries following the replacement of an ineffective therapy with a new, efficacious treatment (Bhattarai 2007; Nosten 2002; Shah 2013). The high burden of drug resistance means that when the treatment failure rates exceed 10%, it is cost-effective to switch to a therapy 10 times the price (Gogtay 2003). This however, still results in an absolute cost increase and greater economic burden for countries.

The emergence of antimalarial drug resistance has also changed the epidemiology of malaria (Figure 14.1). Drug resistance has led to malaria outbreaks, altered the balance of species toward *P. falciparum* (Box 14.1), and contributed to increased private sector care where irrational treatment practices in turn can further drive drug resistance (Wernsdorfer 1994; Mishra 2011).

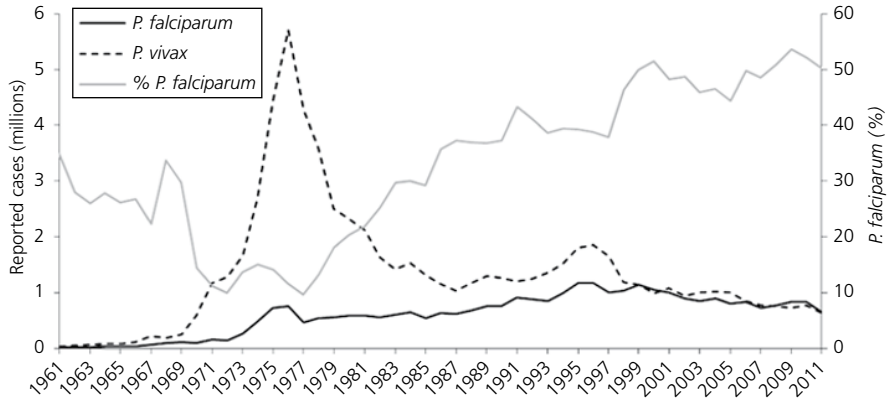

Figure 14.1 Numbers of reported cases of *P. falciparum* malaria and *P. vivax* malaria over the 50 years 1961 to 2011.

Box 14.1 The changing epidemiology of malaria due to drug resistance

The World Health Organization reports a growth in the fraction of *P. falciparum* infections in the Southeast Asia region from 13% in 1977 to 49% in 2000 (World Health Organization 2007b). The changing epidemiology of malaria infections causes concern because *P. falciparum* is associated with increased mortality relative to *P. vivax*. According to surveillance reports, in 2005 *P. falciparum* constituted 44% of all confirmed malaria cases in India, up from 14% in 1975. Longitudinal research by the NIMR has demonstrated tremendous shifts in *P. falciparum* prevalence, particularly in central India. In 1972, *P. falciparum* constituted 42% of malaria cases in Chhattisgarh and 72% in 2002 (Singh 2004). Similarly, in Madhya Pradesh, between 1986 and 2000 the fraction of *P. falciparum* grew from 37% to 87% in children younger than 14 years and from 16% to 93% in adults (Singh 2004). The authors of these reports implicated increasing parasite resistance to antimalarial drugs as a principal cause of changing transmission dynamics.

Causes of drug resistance

Factors related to emergence

Similar to antibiotic resistance, antimalarial drug resistance is an evolutionary response to the selection pressure induced by drugs (White 2004). As the use of antimalarial drugs has increased, so has drug pressure leading to a control paradox: The more drugs are used, the quicker we lose the drugs. The loss of sensitivity can be due to the emergence of *de novo* resistance or the spread of existing drug-resistant strains (White and Pongtavornpinyo 2003). The biological basis for the emergence of drug resistance has been genetic mutations in the drug target, a drug transporter, or both, among identified mechanisms to date. The ability of novel mutants to compete and increase their share of the parasite population is determined by the intensity of drug pressure with, additional factors acting as modifiers of that pressure, including mutation fitness cost, drug half-life, and transmission intensity (Antao and Hastings 2011; Wernsdorfer 1991). Drug half-life is a particularly strong selective pressure because drugs with long half-lives remain in the patient at sub-therapeutic levels for up to several months (Hastings 2002).

Finally, there are differences among parasite species in their propensity to develop drug resistance. Both major parasite species, *P. falciparum* and *P. vivax*, have demonstrated drug resistance in multiple geographic areas to multiple classes of compounds (Price 2007). *P. vivax* tends of have high levels of anti-folate mutations in the absence of any selective pressure, which renders it naturally less sensitive to drugs such as sulfadoxine–pyrimethamine. In *P. vivax,* the emergence of drug resistance has been tempered by the standard therapy of chloroquine plus primaquine acting as a combination treatment of sorts, given the latter has schizonticidal activity against this species. Gametocytes of *P. vivax* also appear simultaneously with the asexual stages in the blood stream infection, which decreases the selection of resistant clones as substantial transmission occurs prior to treatment.

Drug resistance in *P. falciparum* is more extensive and intensive. The two main reasons for the affinity of the species to develop drug resistance are the greater biomass of *P. falciparum,* where higher parasite densities increase the probability of random mutations, and a robust sexual stage that develops after the onset of clinical symptoms and persists after treatment because it is not susceptible to schizonticidal drugs. However, other mechanisms, such as hypermutability and replication error rates, may play a role (Rathod 1997). Unless otherwise specified, our discussion of drug resistance will generally refer to *P. falciparum*.

Mechanisms of resistance

Known mechanisms for antimalarial drug resistance fall into two general categories: mutations in the drug target and mutations in drug transporters (Le Bras and Durand 2003; Petersen 2011). The former decreases binding affinities of the drug and its ability to inhibit the target's activity. Examples include mutations in dihydrofolate reductase *(dhfr)* for cycloguanil and pyrimethamine, dihydropteroate synthase *(dhps)* for sulfadoxine, and cytochrome *b* for atovaquone. The last mechanism includes both point mutations in drug transporters, which may inhibit access to the drug target, and copy number or transcription variation, which may increase drug efflux. Examples include mutations in the chloroquine resistance transporter *(pfcrt)* for chloroquine resistance and an expansion of the multidrug resistance ATP-binding cassette transporter *(pfmdr1)* for mefloquine resistance.

Multidrug resistance

The *P. falciparum* multidrug resistance protein 1 *(pfmdr1)* is a 160-kDa transporter located on chromosome 5. The protein product of *pfmdr1* is known as p-glycoprotein homologue 1 *(pgh1)* due to its similarity with the ATP binding cassette transporters found in mammalian cells. Immunofluoresence and Western blot techniques have verified that *pfmdr1* localizes to the parasite's

digestive vacuole, the site of action for a number of drugs including chloroquine and mefloquine (Zarchin 1986; Adagu and Warhurst 2001). Nair and colleagues analyzed *pfmdr1* breakpoints from a single clinic in Thailand and found amplicons ranging from 6 to 35 kb, indicating multiple independent origins for gene amplification (Nair 2007). Gene amplification, increased mRNA expression, and single nucleotide polymorphisms of the *pfmdr1* gene have all been shown to confer increased levels of resistance to multiple structurally unrelated agents (Duraisingh and Cowman 2005). Thus, the locus exhibits true multidrug resistance. Given the aforementioned, it is hypothesized that the role of *pfmdr1* as an efflux pump may be responsible for drug resistance; however, it is also possible that indirect mechanisms are important in addition to its role as a drug transporter. Many biochemical and epidemiological studies have implicated *pfmdr1* in the parasite's resistance against various compounds (Duraisingh and Refour 2005; Price 1999; Purfield 2004).

Chloroquine

The discovery of the 4-amino-quinoline chloroquine was one of the most important advances in malaria control. For more than 30 years chloroquine provided a safe, cheap, and effective first-line treatment and prophylaxis. Unfortunately, resistance to chloroquine is now practically universal. Chloroquine-resistant phenotypes are the result of 8 to 10 point mutations in the *pfcrt* gene, with K76T mutation being the key mechanistic determinant. The mutation in *pfcrt*, which is located in the membrane of the food vacuole, is thought to prevent the entry of chloroquine and therefore its subsequent binding of the toxic hemoglobin degradation by-product hematin. Although linkage disequilibrium has been demonstrated between *pfcrt* and *pfmdr1*, the role of *pfmdr1* in chloroquine resistance appears to be minimal (Adagu and Warhurst 2001). It is suggested the *pfmdr1* mutations observed in chloroquine-resistant isolates are compensatory for the detrimental effects of *pfcrt* mutations (Mu 2003). At best, *pfmdr1* modulates resistance to chloroquine.

Sulfadoxine-pyrimethamine

The synergistic anti-folate combination of sulfadoxine and pyrimethamine were among the most widely used antimalarial drugs after chloroquine. Individually, pyrimethamine has been used since the 1930s, but its monotherapy was limited due to the rapid onset of drug resistance and treatment failure. Other antimalarial compounds in this class include proguanil and its derivatives.

The targets of pyrimethamine and sulfadoxine are the *P. falciparum* dihydrofolate reductase and dihydropteroate synthase genes essential to the folate metabolic pathway. The mechanism of resistance is a series of step-wise mutations associated with progressive declines of *in vitro* susceptibility as the ability of the drugs to bind to their targets decreases (Lozovsky 2009). For example, the first *dhfr* mutation observed increases the IC50 by 10-fold, whereas I164L, which is typically seen after three or four other mutations, does so 1000-fold (Andriantsoanirina 2011). Clinical treatment failure in Malawi, the Democratic Republic of Congo, and other countries is strongly associated with the quintuple mutation, which consists of a combination of *dhfr-dhps* mutations (51, 58, 108–437, 540) (Alker 2008; Bwijo 2003).

Mefloquine

In the 1960s, the Walter Reed Army Institute of Research staffed a massive antimalarial development program. Mefloquine, a methanol derivative of quinoline, was one of the first compounds to emerge from this initiative. Due to its long half-life, mefloquine is a popular prophylactic, but its use for treatment has been restricted to South America and Southeast Asia. Elevated *pfmdr1* copy number is the major determinant in mefloquine resistance (Duraisingh and Cowman 2005). Several *in vivo* studies have demonstrated strong associations between increased amplification and a clinical outcome (Table 14.2), including when other clinical predictors such as initial parasite density and age were controlled for in the multivariate analysis. *In vitro* IC_{50} results were also associated with *pfmdr1*

Table 14.2 Studies associating increased *pfmdr1* copy number in *P. falciparum* malaria with clinical outcomes

Location	Drug	Outcome	Day	N	Risk	95% CI	References
Thailand	MQ	PP	3	160	2.60	1.30–5.00	Price 2004
Thailand	MQ	TF	28	160	6.30	2.90–13.8	Price 2004
Thailand	LA	TF	42	207	3.20	1.30–8.00	Price 2006
Thailand	MA	TF	42+	169	5.40	2.00–14.6	Price 2004
Thailand	MQ	TF	42	51	2.30	1.27–4.15	Nelson 2005
Cambodia	MA	TF	42	80	7.96	2.47–25.7	Alker 2007

Abbreviations: CI, confidence interval; LA, lumefantrine–artemether; MA, mefloquine–artesunate; MQ, mefloquine; PP, parasite presence in blood smear; TF, treatment failure; N, sample size.

amplification, corroborating the *in vivo* data (Lim 2009). These studies also displayed *in vitro* cross-resistance with halofantrine, quinine, lumefantrine, and artemisinin, suggesting *pfmdr1* amplification as a common underlying mechanism (Sa 1994; Mwai 2009).

Artemisinins

The endoperoxide artemisinin derivatives (artesunate, artemether, and dihydroartemisinin) appear to be well-tolerated and highly efficacious interventions when combined with other drugs (White 2008). Artemisinin use has been rapidly increasing, and these compounds will become more important as multidrug resistant strains proliferate. Diminished *in vitro* susceptibility has been observed in Southeast Asia, and *pfATPase6* mutations in field isolates associated with elevated IC50s were reported from Senegal and French Guiana by laboratories of the Pasteur network (Jambou 2005). Failures of artemisinin monotherapy at the standard dosage (4 mg/kg) for 7 days and the historical dosage (2 mg/kg) in the ARC trials along the Thailand–Cambodia border has confirmed *in vivo* clinical and parasitological failure due to artemisinin resistance, although the proportion of failures remain low, especially with the higher dose (Dondorp 2010).

Anderson's group demonstrated a genetic basis underlying the delayed parasite clearance rates by studying geographical differentiation and haplotype structure underlying a selective sweep on chromosome 13 associated with this trait (Cheeseman 2012). However, among several putative markers, none so far have been established. This may be due to a multi-locus basis for artemisinin resistance. Another interesting possibility is that the resistance is metabolic and arises from the dormancy of ring stages that simply remain quiescent and therefore unaffected for a period until the drug is eliminated (Cheng 2012; Teuscher 2010). A study has identified a molecular marker for ART resistance on the basis of whole-genome single nucleotide polymorphism (SNP) analysis called *kelch-13 propeller (pfk-13),* and for the first time, *pfk-13* mutations were correlated with artemisinin resistance in Cambodian *P. falciparum* clinical isolates (Ariey 2014).

Evolution and spread

Although selective pressure is a key determinant for the emergence and spread of drug-resistant strains, several other factors are important in understanding this phenomenon. When drug resistance is first detected, two questions arise: where did it come from, and why did it arise? Understanding the answers to these questions can help prevent the emergence of drug resistance and can help target control efforts to contain the spread of resistant mutants (Plowe 2008).

The emergence of drug resistance can be explained by either *de novo* emergence, which is the novel generation and selection of resistant clones in that locality, or by the spread of already-resistant clones into the locality. The likelihood of either event depends on the drug in question and its mechanism of resistance in the parasite. For example, chloroquine resistance mediated by a series of mutations in the drug transporter *pfcrt* emerged at only a few sites and subsequently spread. In contrast, resistance to pyrimethamine mediated by SNPs in its target, is thought to have arisen more frequently and can easily be induced *in vitro* as well (Vinayak 2010). For mefloquine, researchers have reported 5% of isolates harboring increased *pfmdr1* copy numbers in Gabon, where almost no use of the drug has been recorded, as well as in travelers returning from other countries in West Africa (Uhlemann 2005; Witkowski 2010). This mirrors the results of microsatellite studies comparing the evolution of *pfmdr1* mutations in multiple countries (Vinayak 2010).

These molecular evolutionary studies mirror the natural history of the spread of drug resistance as well. Chloroquine resistance was first detected in Thailand in 1957, in Burma in 1971, and in Northeast India in 1973 suggesting a sequential westward spread aligned with the major pattern of population movement (Clyde 1972; Seghal 1973; Verdrager 1967). For sulfadoxine–pyrimethamine, resistance has been detected at most sites within 5 years of its introduction, and similarly mefloquine monotherapy was associated with the rapid development of drug resistance as well (Table 14.1).

Microsatellite markers

The analysis of microsatellite markers, simple repeating sequences of DNA base pairs, from regions flanking molecular markers under selective pressure has helped researchers to understand the evolution of drug resistance (Orjuela 2013). A key contribution of microsatellite studies has been to help answer the question of where resistance to chloroquine and pyrimethamine arises. By examining isolates from different regions and comparing the diversity of microsatellite patterns, the number of distinct selection events can be estimated. In the case of chloroquine resistance, limited (four or five) distinct worldwide origins were found, but pyrimethamine resistance appears to have arisen in multiple locations (Anderson and Roger 2005; Awasthi 2012). Microsatellites thus also shed light on the role of selective sweeps versus natural selection models for the evolution of drug resistance against particular treatments. In the case of mutant *pfcrt,* there are certain origins that are represented by distinct haplotypes such as SVMNT in South America and CVIET in India (Pati 2007; Vinayak 2003). Interestingly, India contains a mix of each, with the former fixated in the west and the latter in the east, demonstrating the potent spread of drug-resistant strains from two far-away origins.

Effect of transmission intensity

Where is drug resistance most likely to arise? The contribution of transmission intensity to resistance is complex (Nsanzabana 2010). In areas of higher transmission, the parasite biomass is larger, which increases the probability of the generation of a *de novo* mutant. However, the proportion of infections that receive drug treatment in high burden areas tends to be lower, owing to a combination of acquired immunity as well as the generally poorer healthcare systems in such areas, which reduces selection pressure.

Next, more infections in high-transmission areas are polyclonal, and the probability of recombination during vector transmission serves to limit the fixation of resistant alleles. In addition, high immunity can easily mask drug resistance; in Uganda, for example, transmission intensity rather than drug-resistant mutations determined treatment failure rates against chloroquine, sulfadoxine–pyrimethamine, amodiaquine, and a combination of the last two (Francis 2006). In low-transmission areas, the fraction of infections that are symptomatic is higher, and more infections are likely to be treated with an antimalarial drug. The clonality of infections in low-transmission areas is lower, which increases the probability of self-recombination and preserving rare mutations.

Thus, whereas *de novo* mutations are more likely to arise in high-transmission areas, mutations in low-transmission areas are more likely to persist and be selected for. This is well illustrated by the distribution of *pfcrt* haplotypes in different regions of India. For example, more than 30 years after the first report of chloroquine resistance in the country, K76T has reached near fixation in low-transmission south and western India, whereas in high-transmission Orissa in eastern India, nearly a third of isolates remain wild type. The Thailand–Cambodia border, the most drug-resistant area in the world, is also a low-transmission area.

Reversion of drug sensitivity

The fitness of parasite mutations leading to drug resistance was not previously well understood. In the absence of drug pressure, would mutant parasites fail to outcompete their wild-type predecessors? Malawi provides an interesting case study on this question, as well as detailing the utility of molecular surveillance.

Following high rates of chloroquine failure, Malawi responded by becoming the first African country to withdraw chloroquine and establish sulfadoxine–pyrimethamine as its first-line therapy in 1993. Longitudinal surveillance of the chloroquine resistance marker *(pfcrt)* demonstrated a steadily decreasing prevalence of the mutation (Mita 2003; Kublin 2003). The decline of the previously ubiquitous *pfcrt* mutation following the cessation of chloroquine suggested a return of efficacy, and this hypothesis underwent clinical trials confirming the molecular data. However, when isolates were analyzed with the more-sensitive heteroduplex tracking assays, 5% of clones were detected to harbor the *pfcrt* mutation, suggesting that the efficacy of chloroquine upon reintroduction may be short-lived, as these would readily be selected for (Juliano 2007).

Another marker, *pfmdr1,* might perform an analogous role in Southeast Asia, where deamplification was noted following the introduction of dihydroartemisinin plus piperaquine in Pailin, Cambodia (Imwong 2010). In contrast, for anti-folate drug resistance the removal of drug pressure in Peru and elsewhere has not created a reversion to wild-type genotypes, suggesting a lower fitness cost for isolates harboring such mutations. However, the possibility of drug sensitivity reversion outlines a possibility of long-term cycling of certain drugs.

Detection of drug resistance

The monitoring of drug resistance in malaria uses a mix of *in vitro* and *in vivo* approaches. In addition to the choice of technique, the sampling strategy of where to monitor and how often to monitor are also key questions. In the past 10 years, the use of molecular techniques has added new options for monitoring antimalarial drug resistance as well improving existing approaches.

In vivo monitoring

Currently, the gold standard for drug resistance monitoring is the *in vivo* therapeutic efficacy study. In this study design, a cohort of patients are given observed treatment and followed at fixed intervals for 28 to 63 days and monitored for the reappearance of parasitemia (Box 14.2). By controlling the inclusion criteria, drug intake, and assessment methods, many of the interpretation challenges present in an individual case of treatment failure are removed. However, *in vivo* trials are lengthy, expensive, and difficult to conduct. *In vivo* studies are also poorly comparable across sites because of common local adaptations and variations in transmission patterns. Finally, the results from *in vivo* surveillance do not reflect true parasite resistance due to the interfering effects of host immunity, pharmacokinetics, and previous drug intake; rather, they are useful as a measure of drug efficacy in a given area and population. Thus, despite the disadvantages of *in vivo* studies, they represent a valuable measure of possible programmatic effectiveness.

Box 14.2 Outcomes of therapeutic efficacy studies (World Health Organization 2009)

Early treatment failure

- Danger signs or severe malaria on day 1, 2, or 3 in the presence of parasitemia
- Parasitemia on day 2 higher than on day 0, irrespective of axillary temperature
- Parasitemia on day 3 with axillary temperature ≥37.5°C
- Parasitemia on day 3 ≥25% of count on day 0

Late clinical failure

- Danger signs or severe malaria in the presence of parasitemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and
- Presence of parasitemia on any day between day 4 and day 28 (day 42) with axillary temperature ≥37.5°C in patients who did not previously meet any of the criteria of early treatment failure

Late parasitological failure

- Presence of parasitemia on any day between day 7 and day 28 (day 42) with axillary temperature <37.5 °C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure

Adequate clinical and parasitological response

- Absence of parasitemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any failure criteria

The other methods for monitoring drug resistance, which are more direct, include *in vitro* measurements of drug sensitivity and genotyping molecular markers associated with treatment failure. However, *in vitro* and molecular studies are not possible for every drug, for example where metabolism is required or a marker does not exist, and lab results might not reflect clinical results accurately.

Several advances have helped to improve the traditional *in vivo* study. First, the World Health Organization now promotes a common protocol to help ensure data consistency and comparability between studies and regions as well as minimally acceptable study sizes for statistical validity (World Health Organization 2009). The WHO has also developed a data entry sheet for therapeutic efficacy studies, which facilitates double entry, data analysis, and inclusion checks.

Another key advance in efficacy studies is the use of a follow-up period based on the drug of interest. Although 28-days of follow-up is the minimum recommended period for all studies of antimalarial drug treatment from a programmatic perspective, a considerably longer follow-up is important for drugs with long half-lives, where residual concentrations of drug may be high enough even after one month to prevent the detection of emerging resistance. Thus, for sulfadoxine–pyrimethamine, for example, many studies use 42 days of follow-up and 63 days for mefloquine.

In terms of therapeutic efficacy studies for *P. vivax*, the protocol for chloroquine, which has a 28-day follow-up, is quite straightforward: The period is short, which minimizes relapses, and the residual chloroquine should be able to suppress relapses as well (Baird 2004). Here the interpretation of treatment failure absolutely requires blood levels of chloroquine to be obtained to ensure adequate absorption. However, for other drugs, especially those with long half-lives, the method for monitoring their efficacy in vivax malaria will need to be developed.

Genotyping treatment failures

Polymerase chain reaction (PCR) correction is the use of variable antigen sites such as *msp1*, *msp2*, and *glurp* to distinguish between reinfection and recrudescence as the cause of a treatment failure outcome (Snounou and Beck 1998). Many areas do not have the equipment or expertise to conduct

the technique, which usually consists of nested PCR and/or restriction digesting a PCR product. WHO has standardized PCR protocols for classifying the outcomes of therapeutic trials (World Health Organization 2007a). The importance of PCR correction has increased, especially with the advent of longer follow-up durations, as the risk of re-infection then increases.

However, the interpretation of PCR-corrected outcomes is not always straightforward. For example, a program manager might not care whether a treatment failure within 28 days is a recrudescent infection or a reinfection. For them, the frequent reappearance of especially a clinical episode within one month from treatment might represent an unacceptable burden and represent a concern for the public's perception of the quality of care because patients do not care about the differentiation of a repeat episode, especially so close to a prior one.

Parasite clearance

The time after the initiation of treatment until the clearance of peripheral parasitemia is an important secondary outcome of antimalarial treatment, although the time until fever clearance, which is related but tends to precede parasite clearance, was considered more clinically relevant. Parasite clearance times have taken on renewed importance in the era of combination treatment, especially therapies with artemisinin. For treatment failures following ACT therapy, it may not be apparent whether failures are due to decreased susceptibility to the artemisinin component or the partner drug. However, parasite clearance is largely driven by the rapid action of artemisinin. In the Thailand–Cambodia border, the epicenter of drug-resistant malaria, parasite clearance times after treatment with artesunate monotherapy or ACT have progressively increased from an average of less than 24 hours to more 60 to 70 hours (Phyo 2012). The increased parasite clearance time has been followed by ACT treatment failures (although there is partner drug resistance as well), and extended-duration artesunate monotherapy failures (Vijaykadga 2012). Thus, for therapeutic studies of ACT, a secondary indicator of day 3 parasite positivity has been proposed as an indicator to trigger additional studies and containment activities (Stepniewska 2010).

In addition to parasite clearance time, another aspect of parasite clearance that has become important is the rate of clearance, which is the slope of the clearance curve based on reductions from initial parasitemia (White 2011). The parasite clearance time is limited by several factors, including its dependence on the pretreatment parasitemia because clearance is a first-order process, lag time in clearance due to variations in metabolism, and long tails of clearance due to the release of a few sequestered parasites or delays in splenic removal of dead parasites from circulation. The rate of clearance, on the other hand, is strongly linked to the stage-specific killing ability of a drug. Thus, stage-specific resistance, particularly killing of young rings, which form the bulk of parasites in peripheral blood, may or may not be related to the overall clearance time. The artemisinins are particularly potent due to their broad spectrum of activity across parasite developmental stages. Clearance rate curves are obtained by frequent (6 hourly) smears with density counts (Figure 14.2). The slope of the middle of the curve indicates activity.

ACT versus artemisinin failure

The measurement of clearance and response brings up an important debate. Wongsrichanalai argues that program managers care more about ACT failure, whereas artemisinin failure, though important, is a resource-intensive investigation favored by researchers (Wongsrichanalai 2013). ACT failure can be driven by either component, though partner drug failure is more common. Thus, ACT failures are more common, undermine control, and increase the risk of selecting artemisinin resistance by effectively amounting to artemisinin monotherapy. ACT failures can be measured using standard therapeutic efficacy techniques and managed effectively by switching to another partner drug combination. On the other hand, artemisinin resistance may not exist, may not be

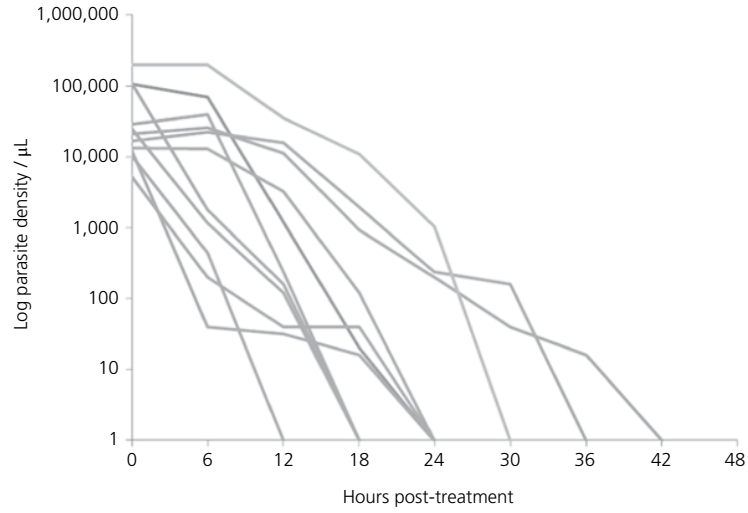

Figure 14.2 Decrease in parasite density following treatment.

transmittable, and requires complicated monitoring to determine its status. Ultimately, it is ACTs that are used in the field, not artemisinin on its own.

Meshnick similarly argues that artemisinin resistance and delayed clearance have been accompanied without clinical or *in vitro* supportive evidence, and they may represent a mild resistance that never progresses to clinical failure, which is a phenomenon similar to that observed in some other parasite systems (Meshnick 2012). Additionally, emphasis on artemisinin resistance has led to the neglect of partner drug failures and has diverted disproportionate resources to an area with an overall small number of cases relative to large burdens elsewhere.

Both authors do acknowledge the importance in artemisinin resistance but wish to downplay the doomsday scenario often used in its discussion. Proponents of studying artemisinin resistance do not believe such trade-offs exist and that both ACT failure and artemisinin resistance must be addressed given the potential consequences if resistance becomes widespread (White 2012).

In vitro sensitivity

In vitro sensitivity testing in malaria is a valuable supplementary tool for monitoring drug resistance developed in the 1970s (Rieckmann 1978). First, decreased susceptibility *in vitro* will precede treatment failures and thus serve as an early warning sign. Second, *in vitro* results directly assess the parasite's response to a drug compared to molecular markers or *in vivo* results, which are more indirect indicators. Finally, such testing is useful for the detailed assessment of antimalarial activity, including the ability to separately assess the components of combination treatments, determine the stage-specificity of drug action, and test the effects of drug–drug interactions including cross-resistance synergism and antagonism.

The limitations of *in vitro* testing include the inability to test certain drugs, the need to adapt parasite strains to culture and the labor and cost associated with it, the risk of strain changes upon culture adaptation, and the variable relationship to *in vivo* experiences. Advances in *in vitro* testing include the availability of free software that can model nonlinear parametric curves to better determine the IC_{50}, as well as a new *HRP-2* antigen test, which allows the measurement of growth inhibition using fluorescence instead of traditional radioactivity (Le Nagard 2011; Noedl 2002). Of note, *in vitro* assays may also be performed *ex-vivo*, that is, immediately after collection without culture adaptation, including a novel stage-specific (ring) assay particularly useful for artemisinin resistance (Witkowski 2013).

Molecular markers

As discussed, resistance to antimalarial drugs arises from two known molecular mechanisms: spontaneous mutations *(dhps, dhfr, cytB),* which affect the structure and activity of drug targets and changes in putative transporters *(pfcrt, pfmdr1)* affecting access to drug targets (Table 14.3). These genetic alterations can serve as markers for clinical resistance. Molecular markers could supplement *in vivo* studies and are potentially powerful tools for such population-based surveillance (Box 14.3).

The surveillance of molecular markers affords numerous advantages. Mutations in key genes will occur before clinical resistance is observed in the field, and therefore molecular markers may provide early warning about any emerging trends (Shah 2008). We can effectively measure these mutations with a number of nucleic acid detection techniques reviewed by Wilson and colleagues (Wilson 2005). Using molecular surveillance, it is possible to test new and withdrawn drugs in addition to existing therapies. For example, in Mozambique, 5 years of molecular surveillance showed that the addition of artesunate to sulfadoxine–pyrimethamine did not halt the progression of *dhfr* and *dhps* mutations, underscoring the risk posed to the efficacy of combination therapies by preexisting resistance to the partner drug (Raman 2010). These are high-throughput techniques and only require a small amount of parasite DNA from a sample such as dried finger-prick blood spots. Thus, sample collection, storage, transport, and analysis are greatly simplified. Additionally, molecular markers can provide insight into the evolution and distribution of drug resistance. Understanding the propagation of resistant phenotypes, especially in relation to local transmission conditions, can help guide rational drug policy (Figure 14.3).

Table 14.3 Antimalarial drugs and associated molecular markers of resistance by species

Species	Marker	Role	Mutation	Drugs affected
P. falciparum	*pfcrt*	Transporter	SNP	CQ*, AQ, QN, PPQ
	pfmdr1	Transporter	SNP	LF, CQ, QN
			CN	MQ*, HF, AS, LF
	pfmdr2	Transporter	SNP	PYR
	pfMRP	Transporter	SNP	CQ, QN
	pfnhe	Transporter		QN
	pfdhps	Target	SNP	SF*
	pfdhfr	Target	SNP	PYR*, PG*
	pfcytB	Target	SNP	ATQ*
	pfATP6	Target	SNP	AS
P. vivax	*pvMDR1*	Transporter	SNP	CQ, AQ
			CN	MQ*, AS
	pvdhfr	Target	SNP	PYR*
	pvdhps	Target	SNP	SF*

Abbreviations: AS, artesunate; ATQ, atovaquone; CN, copy number; CQ, chloroquine; HF, halofantrine; LF, lumefantrine; MQ, mefloquine; PG, proguanil; PPQ, piperaquine; PYR, pyrimethamine; QN, quinine; SF, sulfadoxine; SNP, single nucleotide polymorphism.
* Indicates strong determinant of resistance, otherwise only a modulating role.

Box 14.3 Using molecular surveillance to guide the selection of therapeutic efficacy sites in Cambodia

Monitoring changes in antimalarial drug efficacy is essential, but such studies are resource intensive. In Cambodia, for example, the National Malaria Control Programme (NMCP) can manage to conduct *in vivo* studies at only two or three sites per year because of limited funds and trained staff. In Chumkiri, located 170 kilometers southwest of the capital Phnom Penh, local health center staff observed that falciparum malaria patients treated with artesunate–mefloquine often returned within weeks with recurrent fever and parasitemia. The possibility of artesunate–mefloquine resistance in Chumkiri was particularly alarming because artesunate–mefloquine resistance was thought to be confined to border areas. Chumkiri had not previously been a sentinel site for antimalarial efficacy monitoring by the NMCP, and therefore no *in vivo* data existed.

With the support of the US Naval Medical Research Unit and the Pasteur Institute of Cambodia, *pfmdr1* assays were routinely performed in Cambodia by the NMCP (Shah 2008). This molecular surveillance was high throughput and performed in a central laboratory on dried blood spots, which were easily collected in the field. Thus, samples were quickly collected from Chumkiri, and elevated *pfmdr1* copy number gave credence to the possibility of ACT resistance.

Based on this, a clinical trial was launched in 2006 to evaluate *in vivo* artesunate–mefloquine efficacy in Chumkiri, and a high risk of treatment failure was confirmed (Rogers 2009). Molecular markers can help target *in vivo* studies to where they are needed the most.

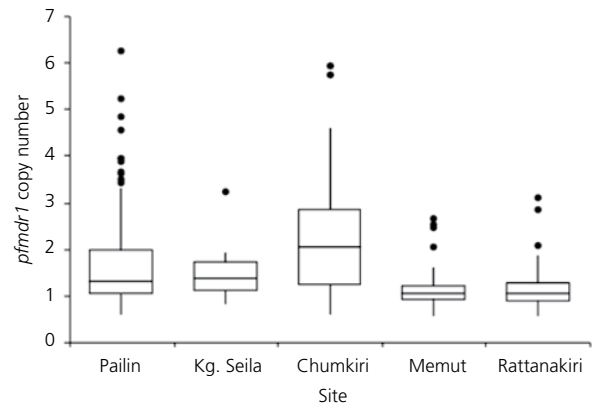

Figure 14.3 The figure shows *pfmdr1* copy numbers at several sites in Cambodia: Pailin, Kampoong Seila, Chumkiri, Memut, and Rattanakiri.

Real-time PCR especially has allowed the high-throughput screening by minimizing steps such as gel electrophoresism which are time-consuming. Large capital costs for the instruments and the necessity for well-trained personnel represent logistical challenges in establishing molecular surveillance. Fortunately, the prices for real-time PCR machines have decreased from US$80,000 to about US$30,000 or less. In addition, it is possible to envision a regional, or at least national, program operating with only one epidemiologist and a technician.

Still, several other limitations exist, including the need for improved sensitivity and specificity, heterogeneous relationships between markers and outcomes even in the same region, and the inability of policy makers to interpret molecular data. The principal obstacle in employing molecular markers, as representing indirect evidence, is to ascertain correlations with *in vivo* results across a wide range of epidemiological settings. In addition to monitoring existing markers, techniques for discovering new molecular markers include genome-wide association studies and high-resolution melting techniques (Timmann 2012; Daniels 2012). Genome-wide association studies are expensive, but they also provide information on population structure and recombination rate, and they

identify loci under selection. The latter is limited to target genetic loci but can identify novel SNPs as well as parasite genotype, and it is cheaper and easier to perform.

Minority variants

An increasingly important concept is within-host diversity of infections. Most parasite infections, even in low-transmission settings, tend to be multiclonal. Although PCR is sensitive, it is thought to only detect clones that comprise at least 20% of the total population (Liu 2008). Thus, variants less than 20% would fail to be detected. This has led some researchers to question the utility of PCR-correction given the risks of misclassification where clonality of infections is high, even terming its use "molecular malarkey" (Juliano 2009). Because drug resistance can vary among these subpopulations, minority variants have a clear clinical relevance. With selection due to a treatment, drug-resistant minority variants become majority variants after treatment and cause treatment failure.

Thus, there are two primary implications of our inability to detect minority variants with PCR. First, in the surveillance of molecular markers of drug resistance, the prevalence of resistant genotypes may be underestimated. Second, in genotyping treatment failures to confirm recrudescence, we risk over-estimating re-infections when we mistake the selection of a minor variant after treatment as a distinct clone because it was unobservable in the pretreatment sample. A key advance in studying and understanding the importance of minor variants has been the development of heteroduplex tracking assays (Juliano 2009). Heteroduplex tracking assays use a probe that is sensitive to small changes in base pairs, the products of which are then resolved using capillary electrophoresis for high-resolution discrimination of band from the digestion of PCR products. Using this technology, clones as small as 1% of the total parasite population can be detected, and new fluorescence-based assays reduce the need for the use of radioactivity for detection (Juliano 2009).

The future of detection

Although many advances have been made in understanding, monitoring, and managing antimalarial drug resistance, several challenges remain.

Point-of-care tests

Nucleic acid–based point-of-care diagnostics are under development. These tests can use whole blood, require only isothermal amplification, and can be read with the naked eye or using simple fluorescence. Although most efforts are directed toward diagnostic ends, tests that detect specific SNPs, for example, could make monitoring drug resistance easier. Cordray and Richards-Kortum reviewed various point-of-care technologies for detecting antimalarial drug resistance (Cordray and Richards-Kortum 2012). Given the short course and low risks of most antimalarial therapy, point-of-care testing to guide individual therapy will remain cost-ineffective in the foreseeable future.

Endpoint assessment

Well-standardized protocols and data management systems have improved the quality of therapeutic efficacy studies, but one glaring source of error remains: microscopy. The quality of microscopy, which determines the primary outcome in these studies, can vary greatly, as do the quality-control systems used to validate their results. In addition to declaring slides positive or negative, microscopic assessments of parasite density also show large variations, though these are less important (O'meara 2005). The advent of day 3 positivity as an important monitoring indicator for emerging artemisinin failure further underscores the need for high-quality microscopy. Thus, new methods to measure the end-points for therapeutic efficacy trials are needed.

Potential technologies to replace microscopy for determining parasite positivity in peripheral blood include flow cytometry and automated image analysis (Frean 2009). Nucleic acid-based

detection methods may also be usable but suffer from potential low specificity due to the detection of material from killed parasites. The ideal technology would enable both detection and counting of viable parasites in addition to being compatible with samples collected from finger pricks whether after transport or at the point of care.

In addition to technological solutions, systemic strategies such as standardized assessments of reader quality and centralized cross-checking solutions for quality control would also enable the continuation of microscopy but may be difficult to implement. The benefit of their use, however, would be as a system-strengthening activity, not just for better classification of trial outcomes but also for the improvement of a broader set of research or program efforts.

Stage-specific resistance

The bulk of work on antimalarial drug resistance has focused, understandably, on asexual stages present in clinical malaria and responsible for symptoms. However, to reduce transmission, the chemotherapeutic control of the sexual stages in *P. falciparum* and the relapsing stages in *P. vivax* are important. Only one class of drug exists for both mature gametocytes and hypnozoites: 8-aminoquinolines. Some research also suggests that the efficacy of primaquine for both indications may indeed be decreasing. It is therefore important to monitor the efficacy of primaquine, yet there remains no accepted means to do so (Baird 2004).

For the assessment of drug activity against *P. vivax* hypnozoites, the major limitations are the need for a long follow-up (1 year in areas with both short- and long-relapse phenotypes) and the inability to differentiate recrudescence, relapse, and reinfection because well-defined genotyping markers, such as those used in PCR-correction for *P. falciparum*, do not exist. Related to anti-relapse resistance is the need to test the efficacy and safety of drugs other than chloroquine when combined with primaquine because anti-relapse efficacy activity may be tied to the choice of partner drug (Baird 2012).

In evaluating the gametocytocidal activity of drugs in *P. falciparum,* the appropriate end-point remains an open question (White 2013). The presence of gametocytes after treatment is considered a proxy for infectiousness. However, the interpretation of gametocytemia is complicated by, first, the ability of submicroscopic densities to cause infection, which requires RNA detection methods, and second, recent evidence of the ability of primaquine to sterilize gametocytes without necessarily clearing them from peripheral blood. The direct assessment of infectivity is conducted by membrane-feeding assays, which requires an insectary and is labor-intensive, precluding larger sample sizes. Developing techniques to assess the efficacy of drugs and sensitivity of these parasite stages will be an important area of investigation, particularly with the renewal of interest in malaria elimination.

Managing drug resistance

Despite the increased challenge posed by antimalarial drug resistance, research and practice provides hope for our ability to tackle the challenge. Coincident with the rise of drug resistance, there has been renewed interest and investment in malaria control and research. Coupled with advances in information technology, genomic science, and an expansion of higher education, we have more technical, financial, and human resources dedicated to its solution than ever before. As a result, the understanding of the mechanisms underlying antimalarial drug resistance, how it evolves, and how it spreads has increased tremendously. Researchers have developed several new tools for monitoring antimalarial drug resistance, have gained more experience implementing varied systems of drug monitoring and more experience constructing drug policies, and have created better support tools for each.

Strategies for prevention

While resistance to drugs may be inevitable, good control policy can help minimize the risk of emergence and spread to increase the shelf-life of available treatments. The management of drug resistance rests on three strategies: reducing drug pressure to prevent the emergence of drug resistance, monitoring the spread of drug resistance to inform treatment policy, and finally creating a pipeline of new antimalarial drugs.

The reduction of drug pressure can be promoted in several ways:

- Rational therapy, where antimalarial drug use is restricted to laboratory-confirmed cases
- Combination therapy that reduces the probability of simultaneous *de novo* or existing resistance mutations
- Drugs with shorter half-lives to minimize the window of selection, so that they do not possess the long tail of subtherapeutic drug levels that can select for resistant clones without inhibiting the infection
- Fixed-dose combinations, improved dosing regimens, and quality-assured antimalarial drugs to minimize the risk of underdosing due to incomplete, inaccurate, or inadequate treatment

An emerging area of concern for reducing drug pressure is the issue of under-dosing. To simplify dosing, age-based, rather than weight-based, dosing is largely used worldwide. Age-based dosing presents the risk though of underdosing or overdosing due to variation in body weight within age categories. Subtherapeutic concentrations of drug contribute to treatment failure and risk fueling resistance. With sulfadoxine–pyrimethamine treatment failure, the strength of the association with underdosing in Kenya increased as drug resistance increased, indicating the complementary roles (Terlouw 2003). Underdosing may be more detrimental in children, where the plasma levels achieved by drugs tend to be lower. Less than one third of Malawian children who received the recommended dose of sulfadoxine–pyrimethamine displayed drug concentration levels above thresholds, predictive of treatment failure (Bell 2011). In relation to the type of resistance, drug levels are thought to be more important in determining the spread of existing drug-resistant isolates rather than the induction of *de novo* resistance.

Antimalarial drugs are more than commodities to be delivered to the sick; they are more appropriately conceived as a critical intervention in a control program, and as such they are only part of a system of case management. This means that the route of antimalarial drug delivery is also important in strategies to prevent drug pressure. For example, a major criticism of the Affordable Medicines Facility for Malaria program, which sought to deliver subsidized ACTs through drug kiosks mainly in sub-Saharan Africa, was its promotion of drug wastage and increased selection pressure because these outlets do not test prior to treatment, nor did they have an incentive to do so (Kamal 2010). Furthermore, most drug kiosks are located in urban and peri-urban settings, and most customers are adults (Cohen 2012). In contrast, malaria incidence is highest in rural areas there and in children. The lack of public health design thinking and an inability to demonstrate improved malaria control led to the closure of the program after several years.

Ensuring a credible pipeline of alternatives is critical. However, the development of alternative drugs alone is not sufficient. Alternative antimalarial treatments should be registered, capable of being manufactured in sufficient quantity, and available at an appropriate price to provide actual strategic depth in choice of treatment. Thus, national control programs should ensure that alternative drugs are registered for use in their country and should survey manufacturers for their ability to supply and for their price, even while the first-line drug is still effective.

In the face of all the aforementioned challenges, ministries of health must respond with evidence-based policy. This policy rests on drug-resistance monitoring using *in vivo, in vitro,* and molecular studies. However, decision making is complicated by variation in resistance between countries and

between regions in a particular country. Concrete, site-specific data could assist national malaria-control programs to efficiently allocate scarce resources. Thus, there exists a need for improved surveillance programs.

Antimalarial drug policy

National drug policies serve several purposes: to promote evidence-based treatment that is safe and effective, to guide case management, and to reduce drug pressure. National drug policies can contribute to the management of antimalarial drug resistance in several ways: by recommending lab-confirmed treatment instead of presumptive treatment or treatment based on clinical diagnosis, by using a combination therapy, and by outlining the detection and management of treatment failures, among others.

Some countries, due to varied patterns in drug resistance, use subnational policies as well. Subnational policies can better allocate resources, such as more-expensive second-line drugs, to where they are needed the most and help stop the spread of drug resistance. However, they do add some amount of program complexity. Myanmar and Peru are examples of countries that use such policies.

Modeling studies suggest that multiple first-line drugs would slow the emergence and spread of resistant strains (Boni 2008). This is a demonstrated concept in agriculture and vector control. However, similar to those disciplines, there would be operational and logistical difficulties in drug procurement, delivery, and health-worker training for implementing multiple first-line drugs in the same area. Overall, monitoring data of antimalarial drug resistance is the most important input in the formation of drug policies, and it is changes in drug resistance that typically trigger the need to modify them.

To prevent the use of oral artemisinin monotherapy, the guidelines of drug policies were strengthened by regulatory efforts to ban its production and sale as well through the national drug regulatory agency. A future need is to harmonize antimalarial drugs listed in essential medicines lists and those recommended in drug policies.

Surveillance systems

Well-defined tools exist for monitoring antimalarial drug resistance, but systems for the routine conduct of drug-resistance studies remain poorly standardized. The choice of how many studies, where, how often, and by whom in many countries is conducted on an *ad hoc* basis depending on interest, availability of trained staff, international collaborations, outbreaks, and, most importantly, funding.

One of the first well-organized systems for such monitoring was developed regionally in East Africa: the East African Network for Monitoring Antimalarial Treatment (EANMAT) in 1997 (EANMAT 2001). In 2001, several countries of the Amazon subregion, with the Pan-AmericanHealth Organization (PAHO), created the Amazon Network for the Surveillance of Antimalarial Drug Resistance (RAVREDA) (Arevalo 2012). Both networks produced common protocols, coordinated resources, shared data, and conducted careful planning of studies. The goals of each were similar: to strengthen the surveillance of drug resistance as well as to promote evidence-based national antimalarial drug policies. In doing so, they produced indirect benefits by bringing together researchers and policy makers who benefited by the former learning the environment and process for policy-making while the latter gained understanding of the science and an appreciation for its relevance to control.

India has a long history of drug-resistance monitoring by the national program along with studies by academia (Box 14.4). With changes in the technology use for drug-resistance monitoring and the introduction of combination treatment, a new system was, however, needed. To address these challenges a joint research and surveillance program – National Vector Borne Disease Control Programme

Box 14.4 History of Antimalarial Drug Resistance Monitoring in India

In 1978, the National Malaria Eradication Programme (now the National Vector Borne Disease Control Programme) created six regional monitoring teams to routinely conduct studies. In addition, the Malaria Research Centre (now the National Institute of Malaria Research) and other organizations supported a wide range of monitoring efforts. Between 1978 and 2007, *in vivo* trials of the first-line and second-line *P. falciparum* treatments, chloroquine and sulfadoxine–pyrimethamine (SP), consisted of 380 studies with 18,944 patients (Shah 2011). The median fraction of treatment failures with SP had increased from 7.7% during 1984–1996 to 25.9% during 1997–2007 in 28 days of follow-up. Indian isolates also demonstrated widespread mutations in *dhfr* and *dhps*, including quintuple mutants in some areas.

Because the efficacy and life-span of artemisinin combination therapies depend largely on the partner drug, preexisting resistance to SP created the possibility of rapid development of resistance to the new combination. Attributing study results to individual components of the combination therapy was difficult, and assessing the efficacy of the partner drug alone was no longer ethical. In 2005, artesunate plus sulfadoxine–pyrimethamine (AS+SP) replaced SP in the national drug policy, and in 2008 it became the first-line treatment for nearly all *P. falciparum* cases in India.

While the efficacy of AS+SP was high (0% median failure, 4% max) in nine 28-day follow-up studies from 2005–2007, the number of sites monitored was still limited given the large size of the country (Ringwald 2005). Thus, in 2009 the joint National Drug Resistance Monitoring System was created based on a series of alternating sentinel sites to provide more areas for monitoring while allowing longitudinal trends (Mishra 2012).

and National Institute of Malaria Research surveillance system – was created. This joint program had several innovations: alternating sentinel sites to provide both longitudinal trends along with widespread coverage, routine *P. vivax* trials to track the emergence of chloroquine resistance, central quality control of slide results and data analysis, genotyping to differentiate recrudescent samples from reinfections, simultaneous measurement of molecular markers of drug resistance, and integration of supplementary studies such as pharmacokinetics and residual drug levels. In the first two years of operating, the benefits included high data quality, national representation of different malaria ecotypes, coordinated use of resources, and pooled data analysis to determine risk factors (Mishra 2012).

The latest global effort for improving the monitoring of antimalarial drug resistance is the World Wide Antimalarial Resistance Network (WWARN) funded by the Gates Foundation (http://www.wwarn.org/). WWARN is focused on global data sharing and conducts an impressive array of work with mapping, data analysis, and standardization and training (Sibley 2007). WWARN also addresses all methods of monitoring: *in vivo, in vitro,* and molecular markers as well as pharmacokinetic studies. However, the potential of WWARN has been limited by its status as a private actor with limited accountability, preventing the submission of data by many researchers and especially national programs. Countries that submit their data readily to WHO, where they have a clear stake, have so far not done so with WWARN. The lack of data limits the utility of the effort, but WWARN has partnered with WHO to improve data depositing (Sibley 2010).

The emphasis on the better use of existing data is important. The pooling of individual patient data across studies allows several advances. First, pooled analysis can provide greater power to detect effects, especially for subpopulations such as children. Secondary analyses on pooled patient data across trials have been conducted on the efficacy of artesunate in high-parasitemia patients. Pooling individual levels also helps ensure analytical consistency in trend analysis, whether over time or across regions. WWARN has also created software that automatically calculates the clearance rate, accounting for the variations in detection limit, lag, and tails (Woodrow 2013).

Conclusion

Antimalarial drug resistance is a challenging problem faced by malaria-control programs. As long as malaria exists, so will antimalarial drug resistance. Thus, basic and operational research are not luxuries but rather are essential to any program success. Even as we tackle current problems and develop new tools, new challenges arise and will continue to do so. We must remain vigilant against this ever-present threat.

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CHAPTER 15

Epidemiology of *Plasmodium falciparum* malaria

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Malaria remains one of the main global health problems, responsible for around 627,000 deaths worldwide in 2012. More than 90% of all deaths occur in the African region, where *Plasmodium falciparum* remains by far the main species responsible for malaria transmission. Malaria transmission is still ongoing in 97 countries, where it contributes to impoverishment of local economies and consumes substantial health resources. Estimating the true social and clinical burden of malaria is not an easy undertaking. The fragile surveillance systems and limited health care access originate vast underreporting. Furthermore, the unavailability of reliable diagnostic methods in many regions of the world and the overlapping symptoms of malaria with other diseases (particularly in children) contributes to frequent misclassification of the disease. Funding for malaria control and research has significantly risen in the last decade, and control programs have increased the use of effective interventions to fight this disease and meet the target goals set by the international community as part of the millennium development goals. Indeed, encouraging downward trends in malaria morbidity and mortality have been reported in many areas of the world. In addition to these achievements, the scientific community has rescued the long-term forgotten ultimate goal of malaria eradication after decades of focusing only in disease control. However, this ambitious goal will not be attained in the short term, and many challenges, including the decline in funding and coverage of key interventions, the emergence and spread of drug resistance, or the dearth of innovative malaria control tools, need to be urgently addressed.

Burden of disease

Malaria is a preventable and curable disease that accounts for a huge burden of disease and death. During 2012, it has been estimated that malaria caused around 207 million clinical episodes worldwide (range, 135,000,000–287,000,000). Approximately 80% of estimated cases occurred in only 17 countries, and the Democratic Republic of Congo and Nigeria account for 40% of the overall global malaria episodes. *Plasmodium falciparum* is responsible for 80% of all cases, ranging from 35% in the Americas to 98% in the African region. *Plasmodium vivax* accounts for 9% of all malaria cases in the world (Table 15.1) (World Health Organization 2013).

Mortality attributable to malaria has decreased since the early 21st century. According to WHO, malaria was responsible for 627,000 deaths (range, 473,000–789,000) in 2012. Around 90% of deaths in the world occur in Africa, most of them in children younger than 5 years and in pregnant women. Seventeen countries concentrate 80% of all global deaths, although two of them,

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Table 15.1 Estimated number of malaria cases and proportion to *P. falciparum* cases by WHO region (2010).

Region	Estimate	Lower	Upper	% *P. vivax*
African	165,000	93,000	242,000	1%
Americas	800	700	1,300	65
Eastern Mediterranean	13,000	10,000	18,000	28
European	0.03	0.02	0.03	—
Southeast Asia	27,000	22,000	33,000	47
Western Pacific	1,000	1,000	2,000	16
World	207,000	135,000	287,000	9

Source: World Health Organization 2013.

Table 15.2 Estimated number of malaria deaths and proportion of deaths in children younger than 5 years by WHO region (2010).

Region	Estimate	Lower	Upper	% <5 y
African	562,000	410,000	722,000	82
Americas	800	500	1,200	27
Eastern Mediterranean	18,000	11,000	31,000	37
European	0	0	0	
Southeast Asia	42,000	26,000	60,000	26
Western Pacific	3,500	2,100	5,200	46
World	660,000	490,000	836,000	77

Source: World Health Organization 2013.

Democratic Republic of Congo and Nigeria, account for more than 40% of deaths in the world. The total number of estimated deaths and deaths in children younger than 5 years by WHO region is depicted in (Table 15.2) (World Health Organization 2013).

There is an ongoing debate within the scientific community regarding the exact mortality figures attributable to malaria. Some argue that these could even be higher, due to an underestimation of malaria deaths in adults and outside Africa, something that most ministries of health in sub-Saharan Africa would strongly disagree with (Murray 2012). One of the main difficulties in estimating malaria deaths stems from the fact that the majority of those deaths occur at home. These unreported cases do not easily reach malaria-control programs, and in addition, methodologies used to estimate the cause of death, such as for instance verbal autopsies, entail a high degree of mis-classification errors. WHO estimates that national surveillance systems detect only 14% of the cases that occur in the world, although this detection rate differs significantly from country to country (World Health Organization 2013). Although estimating the precise malaria burden of disease is a complex issue that indeed deserves further elucidation, current reports are essential for assessing malaria trends within the countries and guide public policy regarding control interventions.

Trends

Malaria morbidity and mortality declining trends since the early 21st century has coincided with an increase in funding for malaria control and research. The scale-up of effective interventions to fight the disease has significantly risen since the early 21st century and could explain the global reduction in the incidence and mortality of malaria. For example, global malaria mortality rates declined around 42% between 2000 and 2012. However, not all countries have shown the same magnitude of reduction in malaria morbidity and mortality. Most of the reduction seen in malaria incidence since 2000 has occurred in countries with lower transmission. Morbidity in countries with very high transmission remains elevated, with little change in the incidence or mortality patterns.

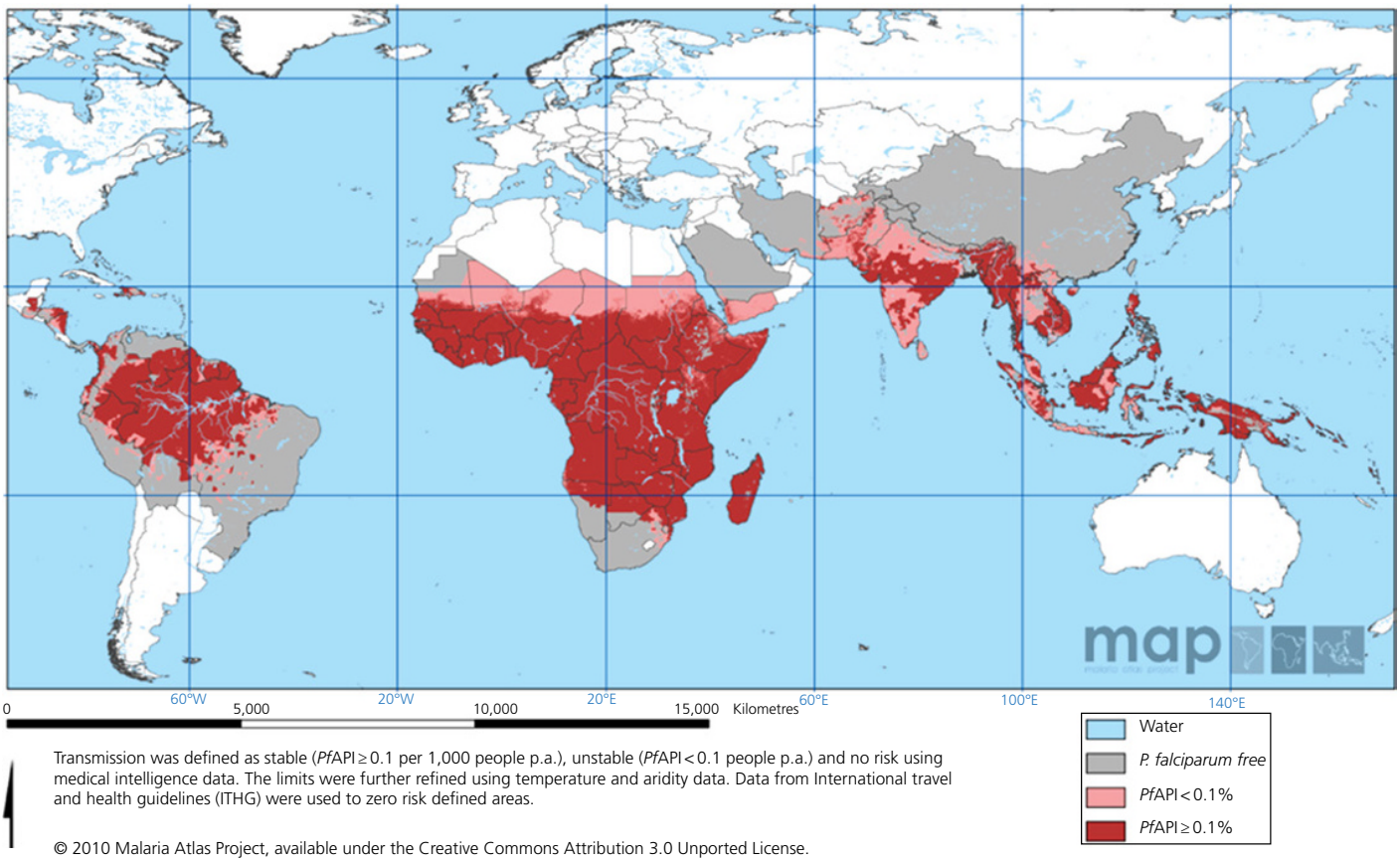
The Roll Back Malaria Partnership and the World Health Organization set the goal of reducing the annual number of malaria cases – malaria incidence – by at least 75% by 2015 (Roll Back Malaria Partnership report 2011). About 52 countries are in line with that goal and will probably succeed in 2015 (World Health Organization 2013). However, meeting this target at a global level with the current decline in funding and without new interventions appears to be a major challenge.

Geographical distribution of the disease

Historically, malaria-free areas in the world have been increasing since the mid-19th century. At that time, the disease was endemic in almost all countries in the world. The malarious endemic areas were greatly reduced during the 20th century as a result of the advent of malaria-control tools, such as chloroquine or DDT, among other factors (Nájera 2011). However, the geographical distribution of malaria-endemic areas increased as a consequence of the emergence and spread of chloroquine resistance and the failure and progressive abandonment of the WHO Global Malaria Eradication Programme (year 1969). In recent years, however, the areas where malaria transmission still occurs have been progressively concentrated between the two tropics, which clearly implies that malaria is not *per se* a tropical disease, but rather has been converted to a tropical disease as a result of human action. This is thought to have occurred as a result of the renewed interest of the international community in malaria control, the launch of global strategies such as the Roll Back Malaria initiative, and the substantial push of international funding for malaria-control interventions. In fact, in recent years several countries have successfully been certified malaria-free by WHO, including the United Arab Emirates (2007), Turkmenistan (2010), Morocco (2010), and Armenia (2012).

According to the World Health Organization data for 2011, malaria is an endemic disease in 104 countries of the world (99 of them with ongoing transmission), where around 3.4 billion people are at risk of being infected. Around 91% of all malaria cases occurred in the African region during 2012, mostly in sub-Saharan Africa. *P. falciparum* is the main species found in Africa and responsible for almost 98% of all cases of malaria in this continent. *P. vivax* is the most prevalent species in the Americas, although *P. falciparum* is also found in the Amazon region and in certain regions of Central America. In Southeast Asia, both *P. falciparum* and *P. vivax* are prevalent at similar rates, whereas in the Western Pacific and Eastern Mediterranean region, P. *falciparum* is the most prevalent species (World Health Organization 2012). Figure 15.1 shows the countries or areas at risk for transmission of P. *falciparum* malaria during 2010.

In the European Region, malaria cases have been declining since 2000. Only three countries reported indigenous cases in 2012, although they have successfully progressed toward the elimination phase: Azerbaijan and Tajikistan, Turkey, and Greece. Although there were only 255



Transmission was defined as stable (*Pf*API ≥ 0.1 per 1,000 people p.a.), unstable (*Pf*API < 0.1 people p.a.) and no risk using medical intelligence data. The limits were further refined using temperature and aridity data. Data from International travel and health guidelines (ITHG) were used to zero risk defined areas.

Figure 15.1 Areas at risk for *Plasmodium falciparum* transmission during 2010 (Malaria Atlas Project 2010).

Plasmodium vivax locally acquired cases in the region in 2012, Greece (certified malaria free since the 1970s) reported 3 and 40 autochthonous malaria cases in 2010 and 2011, respectively, a clear threat of re-establishment of malaria in the country. Since 2009, not a single case of *P. falciparum* malaria has been reported in the WHO European Region (World Health Organization 2012).

Sub-Saharan Africa bears the highest burden of disease and is responsible for the vast majority of deaths in the world. Although great reductions have been achieved in certain countries since 2000, such as those witnessed in Algeria, Botswana, Cape Verde, Namibia, Rwanda, Sao Tomé and Principe, South Africa, Swaziland, or the and Zanzibar (United Republic of Tanzania), many others have failed to fulfill the international targets set by the international community. Nine out of the ten countries with the highest death rates attributable to malaria are found in Africa, and Nigeria and Democratic Republic of Congo are the two leading the list. Highest transmission is still found in West and East Africa (World Health Organization 2013). Figure 15.2 shows the African Region by endemicity level.

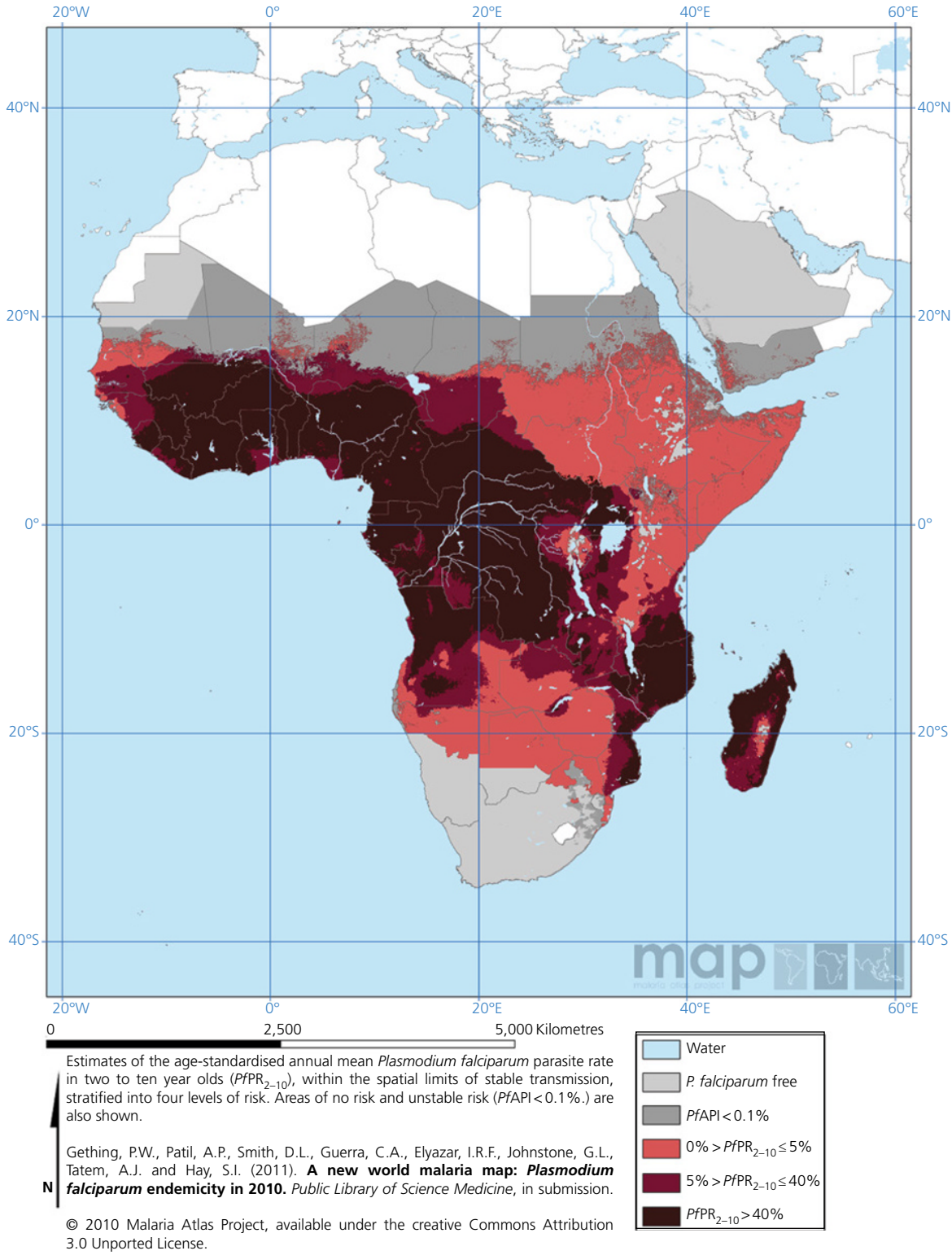
The Americas has achieved great progress in malaria control. Seven out the 21 malaria-endemic countries are in the pre-elimination phase, with very few cases reported in recent years, such as Argentina, Mexico, El Salvador, Costa Rica, Ecuador, Paraguay, and Belize. Brazil and Colombia account for around two thirds of the cases in the region. Haiti, Dominican Republic, and Guyana report the highest percentage of P. *falciparum* cases (World Health Organization 2013).

According to WHO, the region with the highest population at risk is Southeast Asia, although the intensity of transmission there is much lower than that observed in sub-Saharan Africa. *P. falciparum* is most preponderant in Bangladesh, Myanmar, Timor-Leste (East Timor), and India, accounting for the largest number of reported cases and more than a million suspected cases (around half of them due to *P. falciparum*). In the Western Pacific Region, Papua New Guinea is the country with the highest burden of disease. Most countries have also shown downward trends in the reported incidence of cases. *P. falciparum* is the most prevalent species found in the region, especially in Lao People's Democratic Republic (Laos), Papua New Guinea, the Philippines, and Vietnam (World Health Organization 2012).

Chain of transmission and infection cycle

Human malaria is caused by one of the five species of the parasitic protozoa belonging to the genus *Plasmodium: P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi*. All five species differ in the burden of disease they cause, geographical distribution, drug resistance patterns, and clinical symptomatology. *P. falciparum* and *P. vivax* are the two most common species, and *P. falciparum* is the deadliest one. *P. knowlesi* used to affect macaques only, but in recent years, transmission as a zoonosis from macaques to humans has been described, and this species is becoming increasingly important as a cause of human disease, although in limited jungle areas of Southeast Asia, where macaques are commonly found. No direct transmission of *P. knowlesi* from human to human has been described yet.

The parasite gets into human blood through the bite of a female *Anopheles* mosquito. The mosquito is also called a vector, because it transmits the parasite from human to human, acting only as a carrier. Once in the blood of the infected person, the parasite infects liver cells, where it multiplies and grows (liver stage) until a large number of merozoites are released into the blood stream, where they infect red blood cells (blood stage). Gametocytes start developing in red blood cells in capillaries after the invasion by merozoites, usually only after clinical symptomatology has started. The parasites can be found at different stages inside the human body. The main ones are sporozoites,



Estimates of the age-standardised annual mean *Plasmodium falciparum* parasite rate in two to ten year olds ($Pf\text{PR}_{2-10}$), within the spatial limits of stable transmission, stratified into four levels of risk. Areas of no risk and unstable risk ($Pf\text{API} < 0.1\%$.) are also shown.

Gething, P.W., Patil, A.P., Smith, D.L., Guerra, C.A., Elyazar, I.R.F., Johnstone, G.L., Tatem, A.J. and Hay, S.I. (2011). **A new world malaria map: *Plasmodium falciparum* endemicity in 2010.** *Public Library of Science Medicine*, in submission.

Figure 15.2 *Plasmodium falciparum* distribution in Africa by endemicity level for 2010 (Gething 2011).

when they are free in the blood after being injected by the mosquito; schizont and merozoites within the liver and in the blood; and gametocytes in the blood, which are sexually differentiated stages picked up by the mosquito during the blood meal. Inside the mosquito, the parasite also adopts different stages during a cycle of growth (sporogonic cycle): gametes, ookinetes, oocysts, and finally sporozoites in the salivary glands, which are injected into the human, perpetuating the life cycle (Figure 15.3).

The incubation period is defined as the time between infection (sporozoite inoculation by an *Anopheles* mosquito) and the appearance of symptoms of malaria, fever being the most common. This should be distinguished from the pre-patent period, which covers the time between infection and the presence of detectable parasitemia (with parasitological diagnostic tools, such as thick blood smear, rapid diagnostic tests, or polymerase chain reaction (PCR)-based methods). The incubation period depends mainly on the species, and it usually ranges between 7 and 30 days, although much longer incubation periods (including years) have occasionally been described for *P. vivax* or *P. malariae*. *P. falciparum* has the shortest incubation period of all species and *P. malariae* has the longest one.

Malaria endemicity and malaria transmission

Malaria endemicity measures the amount and trends of malaria-related disease in a specific region. It is intrinsically related to the intensity of malaria transmission and depends upon it. It is critical to measure and understand the determinants of malaria transmission in order to identify populations at risk of infection and establish the best control strategies for a specific area. However, there is an ongoing controversy within the malaria scientific community on the best method to measure malaria transmission. There are different classifications describing malaria endemicity based on different indicators (Hay 2008):

- the entomological inoculation rate (EIR)
- proportion of children (2–9 years) with enlarged spleens
- proportion of people with laboratory-confirmed malaria infection (parasitemia rate)
- malaria stability
- number of confirmed malaria cases per 1000 population per year, or annual parasite incidence (API).

Routine case surveillance (i.e., incidence of cases, API), only detects febrile patients who visit government health facilities. It requires, in addition, accurate diagnosis and reporting to the lowest health-system level and does not efficiently differentiate between locally acquired and imported cases. Routine case surveillance is thus particularly inaccurate in countries with poor public health systems and/or a large private health sector.

Some could argue that endemicity or transmission intensity classifications have become old fashioned and lack practical use, although they can provide certain guidance to public health authorities regarding best control interventions in a particular setting.

A review of the methodologies used in the past to monitor and classify transmission may help researchers understand the limitations of these methodologies to deliver in areas where transmission has substantially decreased and malaria is no longer a public health problem. Historically, the first malariometric indicators used relied on the identification of palpable enlargement of the spleen ("spleen rates") among population samples of a specific age group (children 2–9 years of age) in malaria-endemic areas. This method focused on the clinical manifestations of malaria infection on the human population. It was then suggested that measuring asexual malaria parasites in peripheral blood by microscopy would provide increased specificity for malaria infection (Metselaar 1959), although this method was more invasive and varied depending on the season when the survey was

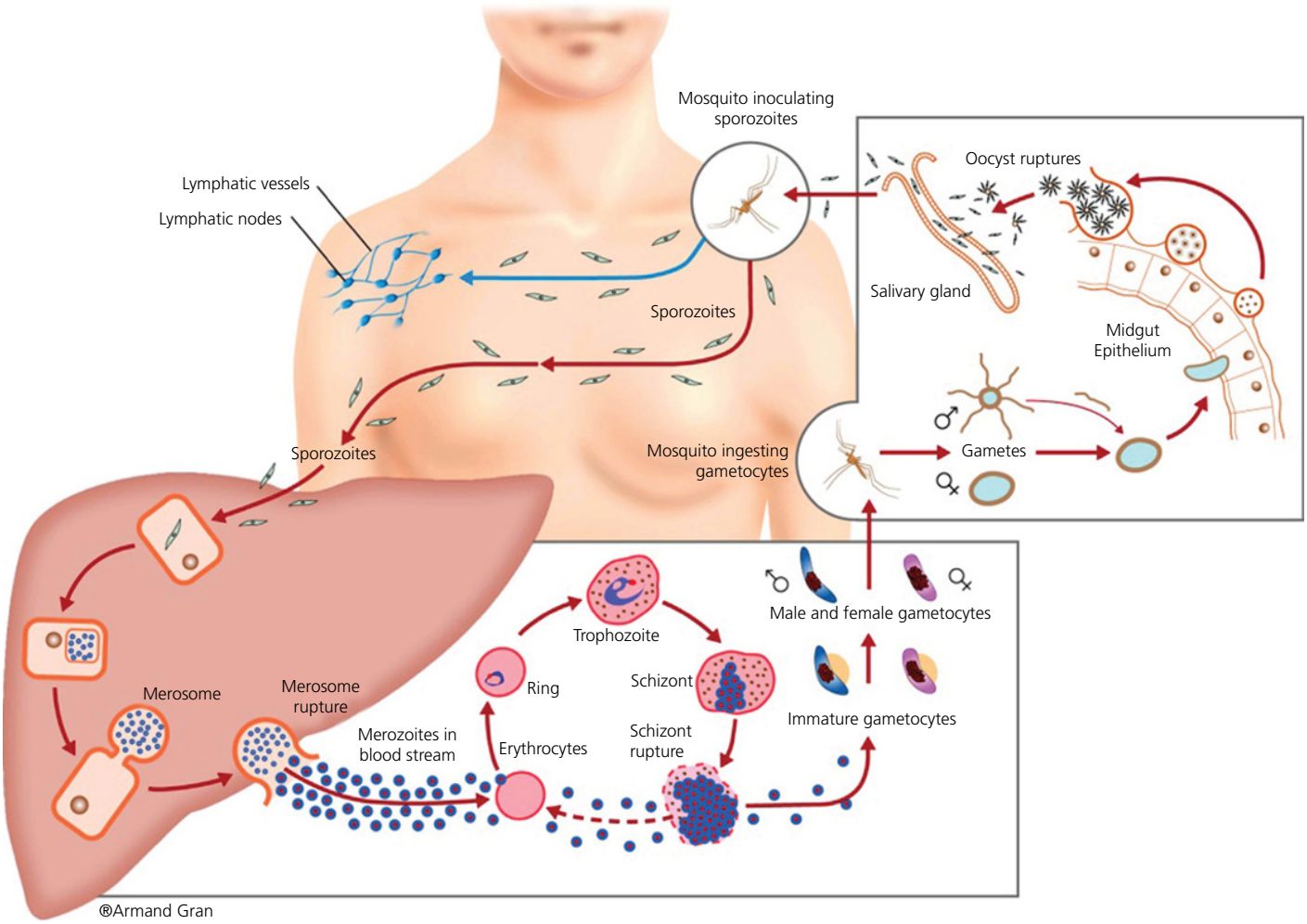


Figure 15.3 *Plasmodium falciparum* life cycle.

conducted. Based on both the parasite and spleen rates, a commonly used classification of malaria endemicity (World Health Organization 2006) uses the following definitions:

- Holoendemic region: Intense transmission occurs all year long, with very high (>75%) parasitemia rates and "spleen rates."
- Hyperendemic region: There is intense seasonal transmission and high parasitemia and spleen enlargement rates (50%–75%), and immunity does not always entail protection to populations living in these areas.
- Mesoendemic region: These rates vary from 10% to 50% within the population. In these areas transmission at a much lower intensity, originating in seasonal malaria epidemics.
- Hypoendemic region: Parasitemia and spleen enlargement rates occur in less than 10% of the population. Malaria immunity does not confer protection, and the population is susceptible for malaria epidemics.

Another commonly used classification differentiates geographical locations as stable or unstable. Stable malaria occurs when populations are constantly exposed to a fairly constant rate of malarial inoculations (World Health Organization 2006). This usually means that malaria infection affects children, and certain immunity is acquired at childhood, partially preventing patients from severe or life-threatening disease in adulthood. Although the original classification of malaria stability originated from a vector-dependent method developed by Macdonald (based on the number of feeds that a mosquito takes on a human being during its life; Macdonald 1952), the current use of "malaria stability" is less precise. In general, hypoendemic regions from the classification based on spleen and parasitemia rates are considered places with unstable malaria, and hyperendemic and holoendemic regions are considered to have stable malaria. The original classification of malaria stability had important limitations, including the technical complexity of obtaining entomological-based metrics, ethical concerns related to exposing human beings to malaria infection, and measurement error issues (Garrett-Jones 1964a, 1964b).Some authors have used new and more operational definitions for stable malaria, such as the minimum average of one clinical case per 10,000 population per annum in a given administrative unit (Snow 2008).

One of the most direct measurements of malaria risk and thus malaria transmission is the entomological inoculation rate (EIR). The EIR takes into account the number of parasite-infected *Anopheles* mosquitoes and their ability to transmit disease. The amount of infectious mosquitoes and the fact that some infectious mosquitoes bite more often than others clearly affects the intensity of transmission in humans. These two variables are captured in the EIR, which is an often-used indicator that estimates the number of bites by infectious mosquitoes per person and per unit time (McDonald 1957).Different techniques are used to measure EIR, although one of the most common is the combination of human bait collections capturers or pyrethrum spray catches to calculate the human biting rate and mosquito dissection to calculate the sporozoite rate (the fraction of vector mosquitoes present and biting that are considered infectious). In fact, this rate is the product of the human biting rate and the sporozoite rate (Kelly-Hope 2009). It is affected by all environmental and climatic factors, which determine the sporogonic cycle and thus the sporozoite rate.

Another entomological indicator that measures efficiency of malaria transmission is the vectorial capacity. This concept, also developed by Macdonald and colleagues in the late 1950s, takes into account the density of vectors in relation to density of hosts, the proportion of vectors feeding on a host divided by the length of the gonotrophic cycle in days, vector competence, daily survival of vectors, and extrinsic incubation period. Although vectorial capacity conceptually captures the main vector-related determinants for malaria transmission, some of the variables associated with it are not easy to obtain. In general, entomological-based metrics to account for malaria transmission entail considerable technical complexity, thus limiting their use.

New methods to assess malaria transmission have been proposed based on the development of antibody responses to given parasite antigens as a measure of exposure in humans (Bousema 2008). Using mathematical models of the percentage of people developing such antibody responses in different age-group categories, a measure of transmission in the given area can be approximated. The bottom line is that none of these methods are currently sensitive enough to detect rapidly important declines in transmission or to monitor ongoing transmission in situations where attack rates have declined substantially.

Indeed, measuring malaria transmission is especially important in those countries aiming at eliminating malaria, where there are important declines in malaria. Analysis of evaluation data from eradication programs as well as closer observations in the field have shown that the point at which malariometric surveys cease to be sufficiently sensitive is reached when parasite rates have dropped to a level of between 1% and 3%, the threshold below which other measures or methods become necessary (Yekutiel 1960). Thus, for this last mile prior to elimination, when control efforts have substantially reduced the burden of malaria, new high-throughput, reliable and reproducible, and highly sensitive methods that do not require the evaluation of parasites in mosquitoes, or the development of clinical consequences of the infection in humans, are necessary to monitor situations of ongoing transmission and, more importantly, to evaluate the impact of tools specifically designed to reduce transmission in such scenarios.

In recent times, innovative methods for monitoring intensity of transmission have been proposed, particularly for areas where malaria incidence has significantly declined. At (very) low levels of residual transmission, there is a tendency toward fragmentation into hot-spots of high transmission coexisting within large areas of little or no transmission (Branch 2005; Moss 2011; Wangdi 2011), and surveillance activities need to be modified if they are to serve as effective tools for further transmission reduction (MalERA Consultative Group 2011b). The force of infection (FOI), which corresponds to the number of infections acquired over time, is another natural measure of transmission intensity. Unfortunately, there are currently no easily applicable tools to measure FOI and delineate areas of high, lower, and no residual transmission. Studies in highly endemic areas have demonstrated that FOI can be accurately quantified using high-throughput genotyping of consecutive samples in longitudinal cohorts (Gong 2012; Mueller 2012), and the molecular force of infection ($_{mol}$FOI, i.e., the incidence of genetically distinct blood-stage infection acquired over time) has been proposed as a gold standard to determine efficacy of novel intervention and evaluate the impact of programmatic efforts in reducing transmission (Mueller 2012). However, determining $_{mol}$FOI is labor intensive and costly and thus not easily applicable to situations where actionable information is required in real time and down a local level. Table 15.3 depicts malaria endemicity levels according to the most frequently used indicators to measure malaria transmission.

Table 15.3 Classification of malaria endemicity levels

Criterion	Hypoendemic	Mesoendemic	Hyperendemic	Holoendemic
Parasite prevalence: spleen rate, children 2–9 years	0–10%	11–50%	>50%	>75%
Entomological inoculation rate	<0.25	0.25–10	11–140	>140
Stability	Unstable		Stable	
Endemicity	Low	Moderate	High	High

Source: Adapted from World Health Organization 2006.

The interplay of naturally acquired immunity and malaria transmission

People living in high-transmission areas, exposed since birth to repeated infections by *P. falciparum*, and surviving up to a certain age (the younger the person, the more intense transmission is) progressively acquire a natural partial immunity to malaria. In areas of stable transmission, severe malaria or malaria-related deaths are rare after 5 years of age, except during pregnancy, when women become temporarily more susceptible. This type of naturally acquired immunity develops with age under continuous exposure to the infection, and it can result in long periods without malaria symptoms in older children and adults (Doolan 2009).

The clinical spectrum of malaria disease is therefore affected by the intensity of malaria transmission. In general, the fraction of asymptomatic cases shifts to younger ages in areas where malaria transmission is increasing. At the same time, severe malaria in highly endemic regions also occurs in the youngest age groups, usually during the first 3 years of age due to a higher exposure to the infective bites and thus parasites, leading to the acquisition of a higher partial immunity against the disease. In fact, it has been described that increasing malaria transmission shifts the proportion of severe malaria and asymptomatic cases to younger ages. Although the age- and exposure-dependent epidemiological pattern of malaria disease is quite well established, the underlying mechanisms of immunity against malaria remain unclear and represent an important research area.

Besides the role of human immunity, intensity of transmission is determined by an amalgam of different factors. These factors could be classified as dependent on the mosquito, environmental and climatic factors, human-related factors, and the implementation of malaria-control tools.

Factors related to the mosquito vector

As the malaria transmission cycle shows, *Anopheles* mosquitos are necessary to transmit the disease from human to human. There are hundreds of described species of *Anopheles* mosquitos, but only around 70 are vectors that are able to transmit *Plasmodium* species, and around 20 of those have local importance in transmission. *Anopheles gambiae*, the predominant vector in Africa, is probably the most efficient vector (Guerra 2008). It belongs to the *Anopheles gambiae* complex, to which other important vectors such as *Anopheles arabiensis, Anopheles merus*, and *Anopheles melas* have also been linked. *Anopheles* mosquitoes have different biting, breeding, feeding, and resting habits depending on the species they belong to, affecting their potential to transmit malaria among humans.

Mosquitoes breed in a wide range of breeding sites. Some mosquitoes prefer to breed next to human settlements, and these are more capable of transmitting the infection. Feeding habits of *Anopheles* mosquitos are also an important determinant for malaria transmission. *Anopheles* mosquitos can feed from humans or from animals. It has been described that *Anopheles* feeding exclusively from humans are more-efficient vectors.

Environmental and climatic factors

Temperature

Temperature changes affect the speed of transmission and the development of the parasite and the mosquito itself. After the gametocyte has been removed from the human blood to the mosquito gut, it undergoes different stages before it becomes a sporozoite, which can then be inoculated back into another human host (Noden 1995). These stages occurring inside the female mosquito are affected by temperature. Thus, the higher the temperature, the faster the parasite would appear to move from one stage to the other, given a minimal temperature below which parasite development does not occur. It has been estimated that *P. falciparum* requires at least 18 °C to go from one stage to the other (Patz and Olson 2006).One could also speculate that global warming may be an important

factor potentially affecting the epidemiology of malaria. Some authors have proposed that lowering the mean temperature in certain geographic areas could favor the appearance of malaria in areas where it traditionally was absent (such as, for instance, higher altitudes), but conflicting evidence has not allowed an adequate confirmation of this hypothesis (Ermert 2012).

Relative humidity

Higher humidity, understood as the amount of water vapor in the air, favors mosquito survival. In some studies it has been demonstrated to be an important factor determining mosquito distribution and therefore malaria transmission (Huang 2011). Some studies in Asia have also shown that low relative humidity (below 60%) inhibits malaria transmission (Yang 2010).

Rainfall

Mosquito vectors breed (reproduce) in water. Therefore, availability of water is essential for mosquito development and thus for malaria transmission. Although great amounts of water can physically destroy breeding habitats, these are recovered soon after the rain has stopped. The existence of rivers next to human settlements has also been associated with increased risk of transmission. This is an explanation for increasing epidemics after severe rain or floods (Castro 2010). However, not all types of water are suitable for all *Anopheles* species. The degree of pollution, vegetation, or streaming and the concentration of salt might influence the breeding capacity of different *Anopheles* species.

Altitude

Increasing altitude has been associated with decreasing malaria transmission in the African highlands (where *P. falciparum* is the most prevalent species) due to lower vector abundance and lower number of infective mosquitoes (De Beaudrap 2011).However, there are studies reporting the existence of abundant *Anopheles arabiensis* at altitudes close to 2000 meters above sea level (Chen 2006). Higher altitudes are associated with lower temperatures, both unfavorable conditions for mosquito survival (Bødker 2003).

Seasonality

Seasonality has been described to affect malaria transmission patterns in hypo- and mesoendemic regions. However, in places where transmission intensity is very high, no seasonal patterns are usually observed, and transmission is observed perennially. At the same time, transmission is very often determined by rainfall, temperature, and humidity. In fact, most seasonal patterns of malaria transmission are mostly driven by rainfall patterns. Seasonal agricultural activities, which entail specific irrigation patterns, also influence the intensity of malaria transmission (Mabaso 2007).

Artificial breeding sites

Uncontrolled urban growth favors the creation of vector breeding sites in a number of manners (Castro 2010). In general, there is usually less malaria in urban settings than in rural settings for a number of reasons. One of them is related to the lower breeding opportunities for mosquitoes. However, in the outskirts of many towns and cities, uncontrolled and disorganized construction provides several breeding grounds for the mosquitoes, favoring transmission (Fournet 2010). Urban agriculture has led to an increasing risk of urban malaria because it provides ideal breeding sites due to the water accumulation in shallows, uncovered wells, human footprints, and irrigation trenches (Afrane 2004). Manmade drains and gutters also provide excellent conditions for mosquito breeding due to the reduced water flow and accumulation of water.

Human-related factors

Migration

Human movements can be associated with increased transmission. Migration from rural (with higher transmission intensity) to urban settings, generally to city outskirts, increase the risk of urban or periurban malaria (Wang 2006). Conversely, when people from urban settings (usually less exposed to malaria) travel to rural areas, the risk of experiencing malaria and severe forms of the disease increases (Carme 1994). This has been described for endemic countries, but it also applies to international travel from nonendemic countries to endemic regions. Human displacements from malarious countries to malaria-free regions could also contribute to reintroducing the disease in the malaria-free regions. In addition, displaced populations are at higher risk for malaria due to their poor living conditions and worse health care access.

Socioeconomic Status

Low socioeconomic status (SES) usually translates into higher risk of malaria. Socioeconomic status can be estimated in different ways. Some of the items commonly used to calculate SES scores in low-income countries are based on asset ownership and household infrastructures. Both factors interplay with the risk of malaria transmission. Being exposed to preventive messages through television or radio is associated with lower risk of infection. Likewise, better housing infrastructure with piped sources of water, isolated rooms (close eaves), or brick floors are associated with decreased risk of infection (Adiamah 1993). Higher SES is associated with better health care access, education, and purchasing power and thus with increased contact with malaria prevention and control tools. At a community level, wealthy neighborhoods have good waste-collection systems and improved water distribution, which minimize the risk of malaria by reducing potential mosquito breeding sites (Fobil 2011).

Malaria control tools

The following malaria control tools have been proven to be effective in reducing the risk of malaria infection.

Insecticide-treated nets

The use of insecticide-treated nets (ITNs) has been promoted by the World Health Organization as one of the key strategies for malaria control. In fact, WHO recommends universal coverage with ITNs for all people at risk for malaria. ITNs have been widely shown to be effective in reducing childhood morbidity and mortality through reducing mosquito bites while sleeping (Lengeler 2004). In other words, ITNs decrease the interaction of the host with mosquitoes during the period when the latter are most infective (at night). In different economic analyses, ITNs were shown to be one of the most cost-effective measures to reduce transmission of malaria. The current WHO recommendation is to use LLINs, which last at least 3 years without the need for periodic re-treatment. It has been estimated that in 2013, around 303 million nets were available to households, covering around 67% of people at risk, which is still below the 80% target set by the Roll Back Malaria Partnership, a malaria initiative launched by WHO, UNICEF, UNDP, and the World Bank.

Indoor residual spraying (IRS)

In Africa, around 8% of the population at risk were protected with indoor residual spraying with insecticide in 2012 (World Health Organization 2013). This strategy, relying on a frequent indoor spraying with long-lasting insecticides of the household walls where mosquitos would rest, acts by reducing the number of mosquitoes present in the households. Pyrethroids are the

most important type of insecticide used for spraying, although DDT, a widely spread insecticide in the past, is still being used in some African and Asian countries despite its potential toxicity and resistance of mosquitoes (Van den Berg 2012). Unfortunately, resistance to pyrethroids has been reported in recent years (Enayatiand Hemingway 2010). In fact, 64 countries have identified resistance to at least one of the four existing insecticide classes (World Health Organization 2012).

Diagnosis and treatment

Prompt diagnosis and treatment of malaria cases annually avert millions of deaths. Until a few years ago, malaria was only diagnosed by direct microscopic examination of intracellular parasites in a stained blood film. This method allows identification of the *Plasmodium* species, quantification of the number of parasites in blood (related to severity), and evaluation of response to treatment. However, this technique requires electricity and specific training, which are not always available in many settings where malaria is endemic, and therefore malaria is treated empirically, without laboratory confirmation. Currently, WHO recommends the use in all endemic areas of rapid diagnostic tests (RDT) (World Health Organization 2010), a new device based in immunochromatography techniques, which detect antigens of the parasite without the need for microscopy. This technique is ideal for remote areas and can also distinguish between *P. vivax* and *P. falciparum*. According to WHO, about 108 million RDT were delivered by manufacturers in 2012, with an expected increasing trend for the near future because parasitological confirmation before initiating specific malaria treatment is now mandatory (World Health Organization 2013).

Parasites have developed resistance to most of the antimalaria drugs developed in the past, such as chloroquine (the most widely used antimalarial drug during the 20th century), atovaquone, and antifolate therapies. In 2004, a new generation of drugs derived from the millenary Chinese plant *Artemisia annua*, the artemisinins, were recommended by WHO as the drugs of choice for *P. falciparum*–uncomplicated malaria, preferably combined with a partner drug (so as to avoid the rapid emergence of resistance to monotherapy) and are collectively known as ACTs (artemisinin-based combination therapy) (WHO Report 2006). By the end of 2012, 79 malaria-endemic countries had adopted ACTs as national policy for first-line treatment, and around 331 million treatment courses had been delivered during that year (World Health Organization 2013). Worryingly, there is already evidence of resistance to these combination therapies in some countries of Southeast Asia, probably related to their initial use as monotherapy (W-WARN Explorer Data).

Chemoprevention

Antimalarial drugs are also used as preventive strategies (chemoprophylaxis). They would not be suitable for continuous use in endemic areas due to the appearance of resistance and potential interference with naturally acquired immunity. However, intermittent administration of antimalarial drugs in the most vulnerable groups – pregnant women and infants – was shown to be an effective strategy to reduce morbidity of both neonates and mothers by maintaining therapeutic levels of antimalarials in blood (Eisele 2012). In fact, 34 out of 45 endemic countries in the African Region have adopted intermittent preventive treatment for pregnant women (IPTp) as national policy by the end of 2012 (World Health Organization 2013). Intermittent preventive treatment for infants (IPTi) has only been implemented in one country (Burkina Faso) despite the efficacy shown in multiple clinical trials (Aponte 2009) and the 2009 WHO recommendation. Another chemoprevention strategy to reduce malaria transmission is mass drug administration (MDA) of antimalarial drugs to populations in a localized geographical area in order to eliminate malaria foci or reduce intensity of transmission in highly endemic regions (MalERA Consultative Group 2011a).

Malaria elimination and eradication

The increased international disbursements toward malaria control, together with political commitment from endemic countries and the international community, has probably had a major impact on the global downward trend of malaria incidence and mortality observed in the last decade. It has been estimated that for 2013, $1.97 billion from international funding has been allocated for malaria-control interventions in endemic countries. Although these figures are far from the estimated $5.1 billion needed for universal coverage of malaria control interventions (World Health Organization 2013), increased coverage of diagnostic tests, treatment, and availability of malaria control interventions such as LLINs or IRS has been observed. These achievements encouraged the Bill and Melinda Gates Foundation and the Global Malaria Action Plan, with the support of WHO and the Roll Back Malaria Partnership, to propose that elimination should be a goal in places where the technical, operative, and financial ability exists to interrupt transmission and prevent its reintroduction. To date, the goal of elimination has been incorporated into the short- and medium-term plans of countries and regions as well as of research organizations and major product development partnerships.

After the failure of the global malaria eradication campaign in 1969, a few countries have succeeded in their goal of eliminating malaria. Malaria elimination, understood as interrupting transmission in a defined region (Box 15.1), is epidemiologically feasible in many settings, especially in those with unstable transmission bordering with nonendemic regions or countries. Conversely, malaria eradication is probably not a feasible undertaking with currently available tools (Alonso 2011).

WHO has established the epidemiological milestones that need to be achieved by countries aiming at malaria elimination and malaria-free certification (Figure 15.4) (World Health Organization 2007). A country's phase determines the most-appropriate objectives and control interventions that should be adopted by the country. For example, countries in control phase should aim at reducing morbidity and mortality, whereas those in the pre-elimination and elimination phases should have the goal of interrupting transmission. Universal coverage with LLINs would be an intervention of paramount importance in the control phase, but it should only be implemented in much localized transmission foci in pre-elimination and elimination phases.

Although the renewed impetus for malaria control is evident and the long-term goal of malaria eradication has been recovered, there are challenges ahead of us that threaten not only this latter goal but also the re-establishment of the disease in malaria-free areas. The increasing drug resistance and the expected reduction in funding for control activities and thus coverage of key interventions or further efforts in research and development for new drugs or vaccines are concerns that urgently need to be addressed. Otherwise, the fight against malaria is far from over.

Box 15.1 World Health Organization definitions of malaria control, elimination, and eradication.

Malaria control: Reducing the disease burden to a level at which it is no longer a public health problem.
Malaria elimination: Interrupting local mosquito-borne malaria transmission in a defined geographical area, i.e., zero incidence of locally contracted cases.
Malaria eradication: The permanent reduction to zero of the worldwide incidence of malaria infection caused by a specific agent. This term applies to a particular malaria parasite species.

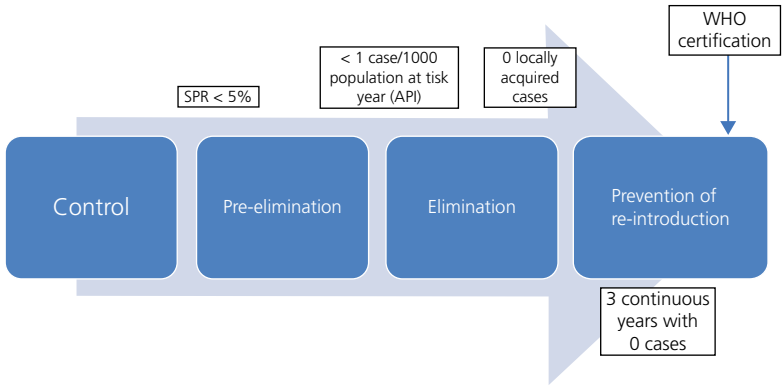


Figure 15.4 Malaria program milestones toward malaria elimination. *API*, annual parasitic incidence *SPR*, slide positivity rate. Adapted from "Malaria Elimination: A Field Manual for Low and Moderate Endemic Countries" (World Health Organization 2007).

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CHAPTER 16
Malaria pathogenesis

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Plasmodium falciparum is the deadliest of the five human malaria parasite species (*P. falciparum*, *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi*). Despite reductions in malaria incidence in many areas (Bhatt 2015), 214 million cases of clinical malaria caused *P. falciparum* occurred in 2015, and 438,000 people died from severe falciparum malarial illness (WHO 2015). *P. falciparum* infection is concentrated in Africa, where 610 million people live in endemic regions (Guerra 2008), with a wide range of malaria transmission intensities.

Adults in endemic areas are partially immune: They experience parasitemia periodically and represent an important infectious reservoir, but they usually carry low parasite densities with few or no symptoms. Children and pregnant women suffer most of the malaria morbidity and mortality in Africa. Understanding malaria pathogenesis and mechanisms of protection will inform the development of more-effective interventions, including vaccines that prevent severe disease.

In this chapter, we review the clinical spectrum and epidemiology of malaria illnesses and the proposed mechanisms of disease and protection, particularly for *P. falciparum* malaria. We argue that the epidemiology and molecular parasitology of severe *P. falciparum* malaria might be explained by virulent parasites with distinct and conserved features, possibly linked to endothelial adhesion, that play a critical role in pathogenesis. We propose that pregnancy malaria represents a model for severe malaria pathogenesis in children, and we discuss the implications of this model on the development of immunity against severe disease.

Malaria illness

Clinical spectrum and mechanisms of disease

Children living in endemic areas

African children with *P. falciparum* infection experience a variety of clinical conditions. Children and adults often carry parasites, including some children with high-density infections, without developing symptoms. In areas of stable transmission, severe disease peaks early in life, often during infancy in the zones of highest transmission (Carneiro 2010). In these areas, most infections occur as uncomplicated disease, with fever and with nonspecific symptoms such as reduced feeding, vomiting, and/or diarrhea that are difficult to distinguish from other childhood infections. Only a small fraction of *P. falciparum* infections result in life-threatening illness (Greenwood 1991). Conversely, severe malaria incidence is spread over a much wider age range in areas of unstable transmission, and a larger percentage of infections develop into severe syndromes (Carneiro 2010; Snow 1997).

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Severe malaria is most commonly caused by *P. falciparum* and consists of a heterogeneous group of syndromes that are associated with high mortality and require prompt treatment: coma, repeated convulsions, severe malarial anemia, and respiratory distress are common presentations in children. African children are more likely to present with a single life-threatening symptom than multiple features of severe malaria. This differs from nonimmune adults, in whom severe malaria caused by *P. falciparum* is characterized by multiorgan disease, often with additional features such as kidney disease and jaundice (Kochar 2006; Sahu 2010). Case-fatality rates in severe malaria episodes in children range from 7.7% to 13.3% but vary by syndrome (Jallow 2012; Marsh 1995; Reyburn 2005). Although severe malaria in children is usually a discrete, rather than overlapping, syndrome (Marsh 1995; Reyburn 2005), the prognosis is worse when multiple symptoms occur together (Jallow 2012; Marsh 1995).

Cerebral malaria is a major neurological complication of falciparum malaria infection. It is characterized by coma not attributable to hypoglycemia, convulsions, or bacterial meningitis, and it is associated with high mortality. Seizures are common in children presenting with this syndrome (Idro 2005). Malaria retinopathy (hemorrhages, white discoloration of retinal vessels, papilledema, and whitening of retinas) is a diagnostic and prognostic sign in African children developing cerebral malaria (Taylor 2004; White 2009). Sequestration of infected erythrocytes, which might reduce microvascular blood flow and/or initiate local inflammation and pathology, is considered an essential event leading to cerebral malaria. In Malawi, autopsies of children clinically diagnosed with cerebral malaria revealed distinct pathological patterns: sequestration of parasitized erythrocytes in cerebral vessels without other pathology, sequestration along with perivascular and intravascular pathology, or no sequestration. In Taylor's study (2004), 23% of children clinically diagnosed with cerebral malaria, including those with no evidence of parasite sequestration in the brain, had other causes of coma and death. Although sequestration is generally believed to play an essential role in severe disease, particularly cerebral malaria, the role of other mechanisms including inflammation and immunopathogenesis remains controversial (Craig 2012).

Severe malarial anemia contributes substantially to malaria-related morbidity and mortality in African children (Marsh 1995; Reyburn 2005). Severe malarial anemia is associated with lower case-fatality rates compared to cerebral malaria, but because severe malarial anemia is much more common it causes a greater total number of deaths (Murphy and Breman 2001). Several mechanisms contribute to severe malarial anemia: erythrocyte destruction either directly due to rupture (of parasitized erythrocytes) or due to erythrophagocytosis or complement-mediated lysis (of nonparasitized as well as parasitized erythrocytes), as well as inadequate reticulocyte production by the bone marrow (Miller 2002). More uninfected than infected erythrocytes are lost as malarial anemia progresses (Jakeman 1999).

In African children, respiratory distress is another common presentation of severe falciparum disease, representing between 7% and 16% of pediatric severe malaria episodes (Marsh 1995). Respiratory distress in children has been attributed to metabolic acidosis (Taylor 1993), which itself is an important prognostic indicator (Marsh 1995; von Seidlein 2012) and can result from several mechanisms: lactate production by oxygen-deprived or hypermetabolic tissue, as well as by the parasite itself; salicylate toxicity, which leads to lactate and pyruvate production but also directly stimulates the respiratory center, leading to hyperventilation (English 1996); as-yet-unidentified acids that contribute to an unexplained anion gap (Dondorp 2004). Respiratory distress in adults is more likely to result from acute lung injury or acute respiratory distress syndrome, though acidosis is independently associated with mortality in this group (Dondorp 2008).

Insights gleaned from clinical studies of severe malaria syndromes or from mechanistic studies in mouse models, especially in experimental cerebral malaria (Craig 2012), have prompted

Table 16.1 Adjunctive therapies studied or considered for treatment of SM, and their impact on mortality.

Therapeutic concept	Proposed mechanism of action	Drug agents or Treatments	Target condition*	Impact on mortality
Suppress inflammation	Limit tissue damage	Dexamethasone; IVIG; anti-TNF mAb; Pentoxifylline; Curdlan sulfate; Rosiglitazone	CM, SM	Mortality unchanged; Rosiglitazone efficacy not yet tested[1–10]
Chelate iron	Withhold iron from parasite; Limit tissue damage	Deferoximine; Deferipone	CM, SM	Mortality unchanged or increased[11–13]
Antioxidant	Limit tissue damage	N-acetylcysteine	SM	Mortality unchanged[14–15]
Anticoagulant	Prevent intravascular coagulation	Heparin; Aspirin	SM	Mortality unchanged[16]
Remove parasites	Reduce parasite burden	Exchange blood transfusion	SM	Mortality unchanged[17]
Volume expansion	Support circulation; Reverse acidosis	Albumin; Dichloroacetate	SM	Albumin may decrease mortality[18–20]
Reduce edema	Reduce intracranial pressure	Mannitol; Dexamethasone	CM	Mortality unchanged[21]
Anti-seizure	Reduce seizure activity	Phenobarbitol	CM	Mortality may be increased[22–23]
Neuroprotection	Prevent ischemic damage in brain	Erythropoietin	Nd	No efficacy data available[24]
Anti-adhesion	Block sequestration of CD36-binding parasites	Levamisole	Nd	No efficacy data available[25]
Nitric oxide	Improve endothelial function	Arginine; Nitric oxide	Nd	No efficacy data available[26–27]
		*CM, cerebral malaria; SM, severe malaria; Nd, no trial results reported in literature		

Adapted from Chandy John *et al* (John 2010).
(Akech[19]; Boggild[10]; Casals-Pascual[24]; Charunwatthana[15]; Crawley[23]; Das[6]; Di Perri[7]; Dondorp[25]; Gordeuk[11]; Havlik[4]; Hawkes[27]; Hemmer[16]; Hemmer[8]; Hoffman[2]; Krishna[20]; Looareesuwan[9]; Maitland[18]; Mohanty[12]; Namutangula[21]; Riddle[17]; Taylor[3]; Thuma[13]; van Hensbroek[5]; Warrell[1]; Watt[14]; White[22]; Yeo[26])

interventional studies of adjunctive therapies to reduce severe malaria mortality (Table 16.1). In general, adjunctive therapies have failed to improve mortality (John 2010). A common strategy for adjunctive treatment has been to limit tissue damage, for example by suppressing inflammation, reducing oxidative stress, or stimulating protective mechanisms such as through erythropoietin receptors in the brain. Other treatments have targeted processes that indirectly lead to tissue damage by reducing parasite burden (overall burden or burden of sequestered parasites), by improving endothelial quiescence, or by reducing intravascular coagulation. Some trials have focused on preventing catastrophic consequences of tissue damage, by reducing intracranial pressure or preventing seizures in patients with cerebral malaria.

Among the adjunctive therapies tested, volume expansion with albumin has decreased mortality (when compared to normal saline) among children with acidosis (Maitland 2005). This suggests that acidosis may play an important role in malaria severity, and acidosis correlates with mortality (Dondorp 2008; von Seidlein 2012). However, fluid expansion with albumin or saline may increase mortality in children with severe febrile illnesses including malaria (Maitland 2011). A few interventions such as iron chelation or seizure prophylaxis can have increased mortality (Table 16.1). Future trials of adjunctive treatments might consider targeting a combination of factors involved in severe malaria pathogenesis, because monotherapies have shown limited or no benefits. Alternatively, the results of these studies may reflect the inherent difficulty of reversing this complex cascade of events.

Pregnancy malaria

Malaria in pregnant women is a major public health problem. Women in endemic areas acquire resistance to malaria after years of exposure, but their susceptibility increases significantly during pregnancy, particularly first pregnancy. The greatest impact is on newborns who are born low birth weight, and this effect of pregnancy malaria is estimated to cause 62,000 to 363,000 infant deaths in Africa each year (Murphy and Breman 2001). Pregnancy malaria can also influence long-term outcomes in infants: in The Gambia, placental malaria was associated with lower weight-for-age, weight-for-length, and BMI-for-age *z* scores in the first year of life, independent of low birth weight (Walther 2010). Pregnant women in areas of stable transmission often develop severe anemia but rarely develop other severe malaria syndromes such as cerebral malaria (Bardaji 2008). These women may also be at higher risk for hypertension or preeclampsia (Muehlenbachs 2006), which can be difficult to distinguish from severe malaria.

In areas of low or unstable malaria transmission, or among nonimmune women, malaria is more likely to be severe during pregnancy, with seizures, coma, and respiratory distress as common features (Luxemburger 1997). Mortality is higher in pregnant women versus other groups with severe malaria (Wickramasuriya 1935). In a Burmese refugee community in Thailand exposed to low malaria transmission, about 1% of mothers died from pregnancy malaria prior to the implementation of antenatal screening and treatment (McGready 2012).

Pregnancy malaria is the best-understood *P. falciparum* syndrome because the placenta provides accessible, abundant material for detailed studies of the sequestered parasites and local immune responses. The histological hallmark of pregnancy malaria due to *P. falciparum* is the accumulation of infected erythrocytes and inflammatory cells in the maternal vascular spaces, called intervillous spaces, in the placenta. Early studies of placental histology by Garnham revealed the kinetics of infection (Garnham 1938). Among susceptible women (whom we now know to be primigravidae), placental parasitemia peaks within a week, when macrophage-rich inflammatory infiltrates appear in intervillous spaces. Placental infections can become chronic, with lower parasite densities, persistent inflammatory infiltrates, and parasite pigment (hemozoin) in fibrinoids and macrophages. Among immune women (multigravidae), parasites are cleared early during infection, averting the inflammatory infiltrate in the placenta.

Chronic and acute *P. falciparum* infections during pregnancy are both associated with adverse outcomes, but their clinical consequences might differ. In Tanzania, preterm delivery is related to a high parasite burden (a marker of acute infection) but not to pigment deposition (a marker of chronic infection) (Menendez 2000). Chronic infection, meanwhile, can lead to fetal growth restriction and low birth weight, as well as maternal anemia (Shulman 2001). Studies (Muehlenbachs 2008; Muehlenbachs 2006) linking pregnancy malaria to gestational hypertension in first pregnancies have implicated chronic infection and placental inflammation as risk factors.

Malaria in non-immune adults

Where malaria transmission intensity is low, severe malaria is common in infected adults. In a low-endemicity area of Tanzania, the median age of cerebral malaria sufferers was 26 years (Reyburn 2005). In older patients, clinical manifestations of severe falciparum disease can differ from severe pediatric disease. Adults often present with cerebral malaria, jaundice, pulmonary edema, and kidney failure; severe malarial anemia and convulsions are less common. In Asia, the case-fatality rate during severe malaria episodes increases with age (Dondorp 2008) and with the number of severe syndromes at presentation. Similar to malaria in children, acidosis and cerebral malaria are major prognostic factors during severe disease in adults.

Travelers from nonendemic regions are highly susceptible: 2% to 16% of *P. falciparum* infections in travelers are severe (Legros 2007). In France, older individuals and natives of nonendemic areas with imported *P. falciparum* malaria had an increased risk of death from the disease (Legros 2007). In another European study, the risk of severe disease increased by 30% per decade of life (Muhlberger 2003). Cerebral malaria is a common presentation. In three series of severe malaria cases in Portugal and in France (Bruneel 2003; Corne 2004; Santos 2012), patients also presented with kidney failure, liver dysfunction, acidosis, severe anemia, and disseminated intravascular coagulation (DIC), in addition to cerebral malaria and other features.

In the absence of immunity, malaria is more severe in adults than in children. The relationship between age and severity of disease was described among transmigrants in Indonesia (Baird 1998): Malaria-naïve adults introduced to a malarious area initially experienced a higher risk of severe malaria compared to their children. Similar patterns occur during malaria epidemics (reviewed in Doolan 2009). This difference in severe malaria risk might reflect age-related differences in immune responses (to all malaria parasites or to virulent parasites), in preferential selection for the growth of virulent parasites in older individuals, or in organ-specific susceptibility to pathology. According to one hypothesis, exposure to other common pathogens increases with age and causes cross-reactive priming of Th1 memory cells, which, when reactivated by malaria antigens, results in a strong inflammatory response and pathological consequences (Artavanis-Tsakonas 2003) that might be most pronounced during initial infections.

Epidemiology of malaria

Malaria epidemiology exhibits great complexity and heterogeneity between sites, in part from different rates of acquiring immunity: Protective immunity is acquired at earlier ages in high- versus low-transmission settings. Also, "malaria immunity" encompasses several forms of acquired resistance that develop at different ages in the same individual: Immunity to severe malaria develops relatively quickly, but immunity that controls parasite density can take several years. Sterilizing blood-stage or liver-stage immunity that prevents infection has not been documented in naturally exposed individuals. Malaria infections occur repeatedly in all age groups, but adults present with low parasite density, and symptoms are mild or absent. Susceptibility is linked to intrinsic host factors, like genetic differences, as well as dynamic factors such as iron status and age. Finally, the genetic diversity of malaria parasites is substantial and might influence the distribution of malaria infection and disease in populations. The interplay between these different factors will determine the outcome of individual infections as well as age patterns of susceptibility and resistance at a population level.

Children living in endemic areas

Understanding the process by which individuals progress from susceptible infants to semi-immune adults can inform vaccine design. Anti-parasite immunity that controls blood-stage parasite burden is acquired slowly over many years. This immunity may require exposure to many parasite variants

that circulate in a community (Bull 1998), or it may depend on developmental changes in the host, such as adrenarche, to be fully effective (Kurtis 2001).

In areas where malaria transmission is stable, malaria risk is low in the first few months of life. In Ghana (Wagner 1998), infants aged 18 weeks or younger were at lower risk for infection. Children younger than 5 months, when infected, tend to be asymptomatic and rapidly clear infection (Franks 2001). This protection might be conferred by passively transferred maternal antibodies, but direct evidence is lacking. Other mechanisms that have been proposed include high fetal hemoglobin levels or the absence of *para*-aminobenzoic acid (PABA) in breast milk (PABA is required for parasite growth).

After the first few months of life, the incidence of infection either plateaus or continues to increase throughout early childhood, suggesting that immunity that controls liver-stage or blood-stage parasite development does not develop during the first few years of life. The incidence of uncomplicated clinical malaria decreases slowly, with a modest reduction over the first 10 years of life, depending on malaria transmission intensity and seasonality (Carneiro 2010).

Severe malaria follows a different epidemiological pattern: The incidence of severe disease peaks and then drops during the first year or years of life, suggesting that children acquire immunity against severe disease more rapidly than immunity against infection or mild disease. Hospitalizations and deaths cluster within a narrow age-window in areas of stable transmission, but uncomplicated malaria events are spread out over a much wider age range (Carneiro 2010). Mathematical models of population-based data on infection and hospitalization have suggested that children become immune to noncerebral severe malaria after 1 or 2 malaria infections (Gupta 1999). Prospective cohort data suggest that immunity to severe malaria is conferred by prior episodes of severe malaria but not mild malaria (Goncalves 2014).

The age at peak incidence of severe disease depends on the transmission intensity: As malaria transmission increases, the age at peak severe malaria incidence decreases (Snow 1997) and more malaria-related hospital admissions occur during infancy (Okiro 2009). Counterintuitively, the overall risk of developing severe malaria in children younger than 10 years might be lower in areas with high rather than low-to-moderate transmission (Snow 1997). Because older individuals are more likely to develop severe malaria in the absence of immunity (see the section Host susceptibility: age, below), the delayed acquisition of immunity in low-to-moderate transmission zones should increase the percentage of infections that progress to severe malaria. Longitudinal birth cohort studies in areas with different transmission intensities might better illuminate this link.

Cerebral malaria and severe malarial anemia also differ in their epidemiology. Cerebral malaria is more common in areas of low transmission, possibly because many of these children fail to acquire protective immunity against severe malaria before the age window when cerebral malaria is likely to occur. Children presenting with cerebral malaria are typically older than children with severe malarial anemia (Jallow 2012); severe malarial anemia risk decreases after age 2 years and cerebral malaria risk increases after age 5 years (Reyburn 2005). An explanation for these differences in age patterns might be that virulent strains that cause cerebral malaria are more rare than those that cause severe malarial anemia, and hence would tend to infect later in life (Gupta 1999). Alternatively, host milieu that is permissive to severe malarial anemia or to cerebral malaria appears (or disappears) at a specific age or phase of host development. Another hypothesis is that cerebral malaria might be related to priming of malaria-specific T cells during early life, causing excessive production of interferon (IFN)-γ and severe immunopathology on reinfection (Artavanis-Tsakonas 2003).

Severe malaria might be related to the presence of parasites with specific virulent features. Cases of severe malaria cluster in time and space, suggesting micro-epidemics (Schellenberg 1998; Snow 1993). Hypothetically, rare virulent parasite strains might spread and cause severe malaria in individuals living close to each other, depending on the geographic range of transmission.

Anti–*P. falciparum* antibody titers in age-matched children with severe or mild disease do not differ in The Gambia, implying that prior infection history is not a key discriminator of severe malaria risk in children (Erunkulu 1992). In an intensive longitudinal study in Tanzania, we observed that most children develop severe disease after their first infections, refuting the notion that immunity develops after one or two infections (Goncalves 2014).

Surprisingly, African infants and toddlers sometimes remain asymptomatic while carrying a heavy parasite burden (called hyperparasitemia). These hyperparasitemia episodes do not carry a risk of near-term mortality (Rowe 2007); the long-term consequences of this syndrome have not been studied. Asymptomatic hyperparasitemia may be a discrete malaria presentation caused by parasites lacking the virulence factors that lead to clinical disease and/or by hosts who are tolerant to parasites and do not develop immunopathological responses. However, many Tanzanian children in our cohort studies who developed severe malaria had previously experienced hyperparasitemia with only mild symptoms, indicating that tolerance to parasites is not a stable phenotype at that age. Taken together, these epidemiological patterns suggest to us that severe disease is caused by virulent parasites that have conserved antigens, and immunity against these parasites develops rapidly, whereas immunity against the parasites causing mild disease develops more slowly.

Placental malaria

Women acquire immunity over many years of exposure that controls malaria infection and disease, but they become susceptible again during pregnancy. In Sub-Saharan Africa, 30 million pregnant women are at risk for *P. falciparum* infection every year, which leads to considerable suffering, including low birth weight and excess perinatal and infant mortality (Desai 2007; Dellicour 2010). In areas with stable malaria transmission, one in four pregnant women have placental infection at the time of delivery (Guyatt and Snow 2004).

The distinct epidemiological signature of pregnancy malaria is its parity-specific pattern: Women in areas of stable transmission acquire resistance over successive pregnancies, so that multigravidas are able to limit parasitemia and avert disease. In high-transmission areas, pregnancy malaria due to *P. falciparum* is estimated to cause 26% of the cases of severe anemia in first-time mothers (Guyatt and Snow 2001) and has also been implicated in gestational hypertension in this group (Muehlenbachs 2006). The parity-specific pattern of pregnancy malaria is less prominent or absent in nonimmune populations, implicating acquired immunity as the explanation for resistance in multigravidas, rather than an intrinsic property of pregnancy that varies with parity.

The decreasing risk of malaria over successive pregnancies is related to the acquisition of antibodies that inhibit adhesion of parasites to chondroitin sulfate A (CSA), which functions as a receptor for parasite adhesion in the human placenta (Fried and Duffy 1996). Levels of these antibodies correlate with improvements in infant birth weight, gestational age (Duffy and Fried 2003), and maternal hemoglobin levels, whereas immunoglobulin G (IgG) levels directed against the variant surface antigens expressed by non-CSA-binding parasites do not influence these outcomes (Staalsoe 2004). Maternal age has also been associated with placental malaria risk: younger women are at a higher risk for acquiring malaria infection during pregnancy, and adolescents may be especially at risk for poor outcomes.

Paradigms of immunopathogenesis and immunity

Pregnancy malaria

Our detailed understanding of parasites and immune responses during placental *P. falciparum* malaria offers a model for studying other malaria syndromes. Parasites that sequester in the human placenta have distinct features. Placental infected erythrocytes (IEs) adhere to the placental receptor CSA but not to CD36 (Beeson 1999; Fried and Duffy 1996), and placental IE surface proteins react

with sera from multigravid women but not men (Fried 1998). Histopathological changes are related to inflammatory processes, especially in first-time mothers who lack immunity (Garnham 1938). Placental malaria induces chemokine production and attracts maternal phagocytic cells to the intervillous spaces. The accumulation of macrophages has been linked to several adverse outcomes: intrauterine growth retardation, low birth weight, and maternal anemia (reviewed in Duffy and Fried 2001). Proinflammatory cytokines (TNF-α and IFN-γ) are usually high during placental infection and have been associated with low birth weight in first pregnancies (Fried 1998; Kabyemela 2008), as have IFN-γ–inducible mediators like the chemokine CXCL9 (Dong 2012). Conversely, interleukin (IL)-10, which attenuates the effects of the strong inflammatory response, has been associated with increased birth weight among primigravidas with placental infection (Dong 2012).

Women in endemic areas uniformly acquire antibodies against placental IE over one or two pregnancies as they develop resistance to pregnancy malaria, and immune sera from multigravid women are cross-reactive with pregnancy parasites collected in Africa and Asia (Fried 1998), suggesting that protective antigens have conserved features around the world. Acquisition of antibody against placental IE is associated with resistance to the severe sequelae of pregnancy malaria (Duffy and Fried 2003; Staalsoe 2004). Thus, immunity to blood-stage malaria may be primarily mediated by antibodies against variably expressed IE surface proteins. These proteins are exported by intraerythrocytic parasites for specialized functions such as adhesion to endothelium and immunoevasion.

Studies indicate that placental IEs express distinct genes and proteins (Francis 2007; Fried 2007), including an IE variant surface protein of the PfEMP1 family called VAR2CSA (Salanti 2003) that is required for adhesion to CSA in some parasite lines (Viebig 2005) and that binds CSA *in vitro* (Gamain 2005). Women acquire antibodies against these different placental parasite proteins over successive pregnancies as they become resistant to malaria (Francis 2007; Fried 2007; Salanti 2003). A program to develop a vaccine based on VAR2CSA or the other proteins expressed by placental parasites is under way (Fried 2013).

Parasite burden and severe malaria in African children

Like pregnant women in areas of stable transmission, naturally exposed children relatively quickly develop immunity that prevents severe malaria (Gupta 1999), suggesting parasite proteins that are targeted by protective immunity have limited diversity. Based on the paradigm of pregnancy malaria, a hypothetical model for severe malaria in children can be suggested: IE with distinct binding properties and expressing distinct surface proteins cause severe malaria syndromes; children become resistant to severe malaria, or to a particular syndrome, as they acquire antibodies against the relevant IE surface proteins; these IE surface proteins have conserved epitopes or have limited diversity worldwide.

The sequestration of *P. falciparum* IEs, which bind to endothelial cells possibly to avoid clearance by spleen, is believed to play a key role in severe malaria pathogenesis. For example, the burden of sequestered parasites in the brain is associated with cerebral malaria in autopsy studies (MacPherson 1985; Seydel 2006; Taylor 2004). The association of organ-specific parasite sequestration to other syndromes is less clear, and therefore might be imputed to other factors such as total parasite burden in the body (see below). However, parasites do sequester in the vascular beds of organs involved in noncerebral syndromes (Figure 16.1), such as lung and bone marrow (Pongponratn 1991; Seydel 2006; Wickramasinghe 1987). Parasite adhesion to endothelium, and sequestration in deep vascular beds, depends on the expression of parasite proteins that are present on the IE surface and that are major targets of naturally acquired immune responses. (For more detailed discussion, see the sections on Parasite adhesion and Surface proteins of infected erythrocytes.)

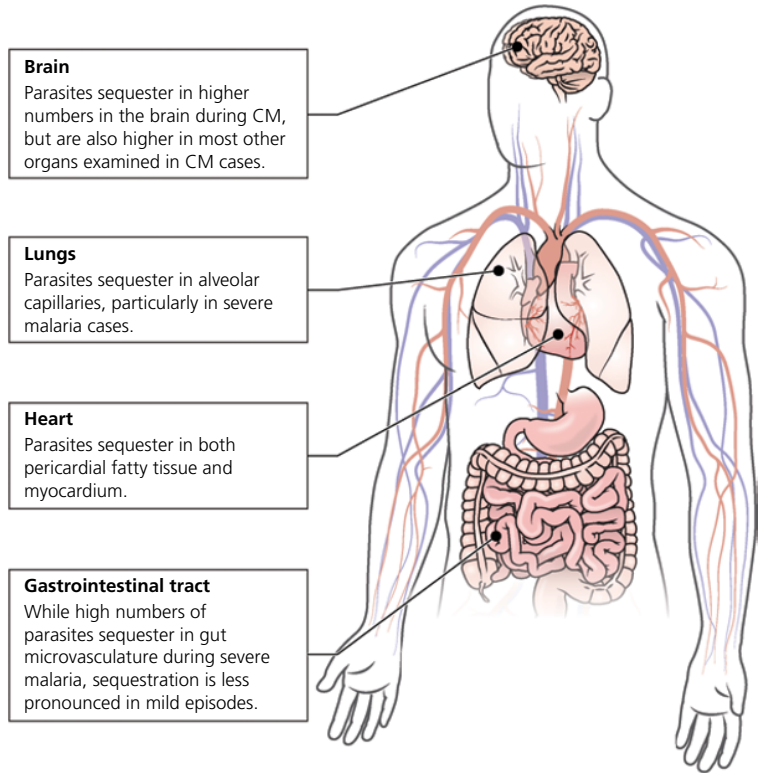

Figure 16.1 Sequestration of *P. falciparum*-infected red blood cells is thought to play a key role in parasite virulence and occurs in several vascular beds in the human host. The histologic appearance and level of sequestration vary between vascular beds and between infections. The level of sequestered parasites in the cerebral vasculature correlates with cerebral malaria risk in several studies of adults and children. In addition to brain, lungs, heart, and gut, sequestered parasites appear in vessels of skin, adipose, kidneys, and other organs.

The total body burden of parasites has been proposed by some to be the major or sole factor determining disease severity during malaria infections. Several studies have quantified parasite biomass either by blood smear or by levels of histidine-rich protein-2 (HRP-2), a secreted parasite protein that has been touted as a better measure of total parasite burden. In some studies, individuals with severe malaria have had higher parasite burdens on average compared to individuals with uncomplicated disease (Hendriksen 2012; Rubach 2012), and a higher parasite burden during severe malaria is associated with higher mortality (Dondorp 2005; Hendriksen 2012; Rubach 2012). However, other studies failed to find a relationship between parasite burden and severity (Manning 2011), and most studies that found this relationship also observed an overlap of parasite levels among individuals with mild versus severe disease. Therefore, other factors such as immunopathology or local parasite burden in vital organs need to be considered as major determinants of disease severity during *P. falciparum* infections.

Balance of proinflammatory and antiinflammatory responses

The inflammatory response during malaria infection may be a double-edged sword, with roles in the control of parasite density as well as the development of disease. In rodent models of malaria, an inflammatory response early during an infection improves control of parasite density, whereas a

delayed inflammatory response occurring late in the course of infection can exacerbate symptoms (von der Weid and Langhorne 1993). In African children, severe malaria syndromes are associated with elevated levels of inflammatory mediators, such as tumor necrosis factor (TNF)-α (Kwiatkowski 1990). Studies in animals (Freitas do Rosario 2012) and humans (Walther 2009) have highlighted a potential role for IL-10–expressing Th1 cells that offer a balance of inflammatory and antiinflammatory responses in order to limit tissue damage and disease.

At early stages of malaria infection, innate immunity plays a crucial role in controlling the exponential growth of parasite density. Cell-mediated immune mechanisms contribute to this resistance and, during infection, macrophage activation by IFN-γ enhances phagocytosis of infected erythrocytes (Ockenhouse 1984). In mouse models, cytokine dynamics in the first hours of blood-stage infection predict the outcome of infection (De Souza 1997). IFN-γ production is important and, at this stage, is produced by innate immune cells, particularly NK cells.

Among African children, low IFN-γ levels increase risk of clinical malaria and higher parasite densities (D'Ombrain 2008). Malaria antigen-stimulated peripheral blood mononuclear cells (PBMCs) from children with severe disease produce IFN-γ less efficiently than PBMCs from children with mild symptoms (Luty 1999). However, excessive IFN-γ might also lead to immunopathology: In mice, IFN-γ is essential for the development of experimental cerebral malaria (Amani 2000). IFN-γ induces the quinolinic acid (QA) pathway of tryptophan metabolism, and QA is neurotoxic, providing a potential link between IFN-γ and cerebral disease (Artavanis-Tsakonas 2003).

TNF-α is another proinflammatory cytokine that may contribute both to parasite control and to disease exacerbation. TNF-α is released by macrophages in the presence of malaria molecules, such as hemozoin and glycosylphosphatidylinositol (GPI). In animal models, TNF-α is necessary to limit parasite density (Jacobs 1996), but high levels of this cytokine have been associated with cerebral malaria and severe malarial anemia in African children (Grau 1989; Kwiatkowski 1990). TNF-α was detected in the brain tissue of fatal human cerebral malaria cases (Udomsangpetch 1997). In a mouse model of cerebral malaria, blocking TNF-α with antibody prolonged survival (Hirunpetcharat 1999). As seen with IFN-γ, moderate levels of TNF-α might optimally control parasite replication without causing host disease.

The complement system, which is an important component of innate immunity, has also been associated with malaria immunopathology. In mice, absence of C5 or blockade of C5a signaling (through genetic deletion or antibody treatment, respectively) prevented cerebral malaria and improved survival (Patel 2008). Both classical and alternative pathways of the complement system might contribute to the pathogenesis of human clinical malaria (Silver 2010). Interestingly, neither pathway is required for activation of the terminal complement pathway in mice that develop experimental cerebral malaria (Ramos 2012). Mechanisms that regulate complement activity are also associated with malaria outcomes in humans. In Kenya, children with severe malarial anemia had lower levels of complement regulatory proteins (CR1 and CD55) on the surface of erythrocytes, which might increase sensitivity to complement lysis and accelerate splenic clearance (Waitumbi 2000).

Based on a model in which pathological processes are triggered by strong inflammatory responses, antiinflammatory agents might prevent tissue damage and disease. For example, a high ratio of IL-10 to TNF-α is associated with reduced risk of severe malarial anemia in Kenyan children (Othoro 1999). Ghanaian children with severe malarial anemia have lower levels of the antiinflammatory cytokine IL-10, compared with children with either cerebral malaria or uncomplicated malaria (Kurtzhals 1998). On the other hand, antiinflammatory effects early in infection might impair mechanisms of parasite control, leading to high-density infections and excess morbidity. In humans, levels of T regulatory cells correlate with accelerated blood-stage parasite growth after experimental sporozoite infection (Walther 2005). In West Africa, the Fulani people, who are less susceptible to malaria compared to other ethnic groups, have functionally deficient T regulatory cells (Torcia 2008).

Taken together, these observations suggest that naturally acquired immunity that prevents malarial disease might be related to a balanced production of immune mediators that both enhance and block the inflammatory process throughout infection. Inflammatory responses in early stages of infection prevent exponential growth of the parasite but are probably harmful when parasite burden is already high. Consequently, functional polymorphisms in host genes related to inflammation, such as those encoding IFN-γ, TNF-α, IL-10, and IL-4, might influence susceptibility to infection and disease. These genetic differences might alter the innate immune response to infection and contribute to the variable clinical outcomes.

Antibody-mediated immunity

Passive transfer of IgG from immune adults in West Africa cleared blood-stage *P. falciparum* infections and alleviated malaria symptoms in children in West and East Africa and in adults in Thailand (Cohen 1961; McGregor 1963; Sabchareon 1991), providing direct evidence that acquired immunity to blood-stage *P. falciparum* malaria relies at least in part on the antibody response. The effector mechanism of protective antibody might be related to blocking invasion of erythrocytes by merozoites; to preventing egress of schizonts from erythrocytes (Raj 2014); to opsonization of merozoites, which leads to clearance by phagocytic cells or complement-mediated damage; to neutralization of virulence factors such as GPI (see the section on Parasite virulence); and/or to inhibition of cytoadherence and consequently reduced sequestration (see the sections on Parasite adhesion and Surface proteins of infected erythrocytes). Cytophilic antibody subclasses, IgG1 and IgG3, correlate with protection from *P. falciparum* disease in humans (Bouharoun-Tayoun and Druilhe 1992; Roussilhon 2007), suggesting a role for phagocytosis. Antibodies that block parasite adhesion in the placenta are strongly associated with protection of pregnant women from malaria, identifying the IE surface as a target of naturally acquired protective immunity for that syndrome.

Other than the special case of pregnant women, the antigenic targets of antibodies responsible for protection against clinical malaria, and more specifically against severe malaria, have not been identified. If protective antibodies that control parasite growth target variant antigens, then clonal antigenic variation might explain the slow development of this form of immunity. In The Gambia, serum from a convalescent child agglutinates IE from the infecting homologous isolate but not from isolates of other children (Marsh and Howard 1986); sera from adults often react with infected cells from all the children. In parallel, Kenyan children with clinical malaria are infected with parasites expressing a PfEMP-1 variant that is not recognized by their preexisting anti-PfEMP1 antibody response (Bull 1998).

Some have argued that the accumulation of these different variant-specific antibodies throughout childhood confers protection from malaria. However, immunity to severe malaria is acquired very early in life and may therefore involve conserved antigens or processes. One possibility is that antibody responses develop against parasite DNA or GPI moieties (which are common to all parasites) and reduce their activity as stimulators of Toll-like receptors and inflammasome components (see sections on hemozoin, parasite DNA and GPI). Another possibility is that specific parasite strains or phenotypes are responsible for severe disease, and these features provide a conserved target for protective immunity. For example, acquisition of antibodies that block the binding of virulent parasites to endothelial cells in vital organs would prevent localized immunopathology and the subsequent cascade of events that lead to severe disease.

PfEMP1 is an obvious candidate antigen owing to its roles in parasite sequestration and antigenic variation. Studies of genetically modified *P. falciparum* with altered PfEMP1 expression suggest that most of the human antibody response is to the IE surface targets PfEMP1 (Chan 2012). VAR2CSA is a PfEMP1 family member with a well-documented role in adhesion and antigenicity of placental IE, and antibodies against VAR2CSA correlate with protection from pregnancy malaria

in some studies but not others. PfEMP1 elements called domain cassette 8 (DC8) and domain cassette (DC13) may be overexpressed in parasites from children with severe malaria (Wassmer 2015) (see the section on Surface proteins of infected erythrocytes). African children acquire antibodies against this element relatively early in life, although more data are needed to demonstrate that these antibodies are related to protection from severe malaria.

Severe *Plasmodium vivax* and *Plasmodium knowlesi* malaria

Plasmodium vivax

P. vivax is widely distributed in South and Central America, Asia, and parts of Africa, and 2.5 billion people live in endemic areas (Gething 2012). *P. vivax* infection has been known as benign tertian malaria (tertian referring to its every other day periodicity), in comparison to the malignant tertian parasite *P. falciparum*. In otherwise healthy individuals, *P. vivax* does not cause severe disease (Anstey 2012; Luxemburger 1997). Despite being a benign disease in individuals without comorbidities, *P. vivax* infection can contribute to severe outcomes among children and adults with co-infections or other precipitating conditions like malnutrition (Lanca 2012). Severe anemia, acute respiratory distress syndrome (ARDS) and multiorgan disease, including acute kidney injury, are increasingly recognized as manifestations of severe *P. vivax* malaria in some areas (Kute 2012; Price 2009; Tan 2008).

Children are at higher risk for severe anemia, which is the main presentation of pediatric severe vivax malaria, but adults can also develop severe anemia. Respiratory distress also occurs, but its incidence varies considerably (Genton 2008); respiratory distress is associated with higher case-fatality rates compared to severe anemia during *P. vivax* infection (Alexandre 2010). Neurological manifestations are less common with *P. vivax* versus *P. falciparum* infection. In Indonesia, coma was 23 times less common in individuals infected with *P. vivax* compared to *P. falciparum*, usually occurring in young adults with low parasitemia (Lampah 2011). Shock, most often associated with multiorgan dysfunction, has been reported (Kochar 2005), but co-infections were not excluded. *P. vivax* is not a common cause of severe disease in pregnant women.

The pathophysiology of disease due to *P. vivax* and *P. falciparum* differs in important ways. *P. vivax* infects only reticulocytes and generates lower parasite biomass compared to falciparum infections. The systemic inflammatory response is greater during *P. vivax* than *P. falciparum* episodes with similar parasite biomass (Hemmer 2006). On the other hand, cytoadherence is much less prominent in *P. vivax*: erythrocytes infected with *P. vivax* adhere to endothelial cells with much lower frequency than *P. falciparum*–infected erythrocytes (Carvalho 2010), and microvascular obstruction due to sequestration of *P. vivax*–infected erythrocytes is minimal (Anstey 2012). Autopsy of an Indian woman with rapidly fatal vivax respiratory distress found no evidence of parasite sequestration in the lungs (Valecha 2009), and placental sequestration of *P. vivax* was not observed in a survey of 175 placentas from Thai women treated for pregnancy malaria, including 83 with *P. vivax* (McGready 2004).

Plasmodium knowlesi

P. knowlesi is an emerging cause of malaria in Malaysia and Southeast Asia. Previously, *P. knowlesi* infection was thought to be limited to macaques for all but the most exceptional cases; it is usually asymptomatic or results in only mild disease in these monkeys. Natural human infections have been sporadically diagnosed since the 1960s (Fong 1971), until an investigation in 2004 of atypical *P. malariae* infections led to the recognition that a large number of Malaysian patients, mostly adults, are infected with *P. knowlesi* (Singh 2004). Retrospective studies subsequently determined that several cases of malaria previously attributed to other *Plasmodium* species were instead caused by *P. knowlesi* (Lee 2009).

The epidemiology of *P. knowlesi* is related to its vector distribution, and most likely transmission is zoonotic. The majority of patients with *P. knowlesi* infection do not develop major complications (Daneshvar 2009), although *P. knowlesi* can cause severe disease and fatal cases have been reported (Cox-Singh 2010). *P. knowlesi* has a 24-hour periodicity, which can lead to rapid growth. Parasite density is related to disease severity (William 2011). The most common severe sequelae are respiratory distress (Kantele and Jokiranta 2011), probably related to pulmonary etiology rather than metabolic acidosis (Daneshvar 2009), and kidney failure (William 2011). Neurological symptoms are rare (Daneshvar 2009) despite sequestration in the brain, as well as heart and kidneys (Cox-Singh 2010). Severe malarial anemia, another important presentation of severe *P. falciparum* malaria, is not observed during *P. knowlesi* infection (William 2011). The data on *P. knowlesi* infection in children are scarce: *P. knowlesi* infection is most often associated with uncomplicated disease; moderate anemia has been observed in children (Barber 2011).

The distinct features of *P. knowlesi* might provide insights into severe malaria during *P. falciparum* infection. Although *P. knowlesi* sequesters in several vascular beds including the brain, syndromes like cerebral malaria and severe anemia typical of *P. falciparum* have not been associated with *P. knowlesi*. One possibility is that these *P. falciparum* conditions share a common etiology that is not evoked by *P. knowlesi*. Similarly, respiratory distress due to *P. knowlesi* is not related to acidosis as is seen in children with respiratory distress due to *P. falciparum*; instead, the respiratory distress of *P. knowlesi* is thought to have a pulmonary etiology, most likely related to hypoxemia from acute lung injury (William 2011). Longitudinal epidemiological studies are needed that describe the pattern by which severe *P. knowlesi* disease develops over successive infections, and the acquisition of clinical immunity, in order to compare and contrast with *P. falciparum* infection and disease, and give better insights into pathogenesis.

Host susceptibility

Factors influencing host susceptibility are shown in Figure 16.2.

Host genetics

Host genetics influences severe malaria risk and may explain in part why only a subset of children in areas of high transmission develop severe malaria. Genetic factors associated with a variety of malaria resistance phenotypes and the likely mechanisms underlying these associations have been discussed in great detail in Chapter 17 by Tom Williams. Of note, some factors such as sickle cell trait are associated with protection against all different severe malaria syndromes (May 2007), whereas other polymorphisms influence the risk of individual syndromes, such as the effect of α-thalassemia to protect selectively against severe malarial anemia (Wambua 2006). Although different factors might modify the risk of different syndromes, they do not fully explain why African children of the same age, living in the same village, can present with very distinct forms of severe malaria.

The effect of these different polymorphisms on malaria phenotypes other than severe malaria can also provide valuable insights into pathogenesis. Hemoglobinopathies influence disease severity (Taylor 2012), but they do not seem to influence *P. falciparum* prevalence. Additionally, sickle cell trait is associated with lower parasite densities during malaria infection (Williams 2005), suggesting that protection associated with HbS might therefore involve an overall reduction in parasite burden, which would explain its syndrome-transcending effect. On the other hand, Southeast Asian ovalocytosis (SAO) (Allen 1999) and α-thalassemia reduce severe malaria risk without modifying parasite levels (Fowkes 2008), which supports the concept of

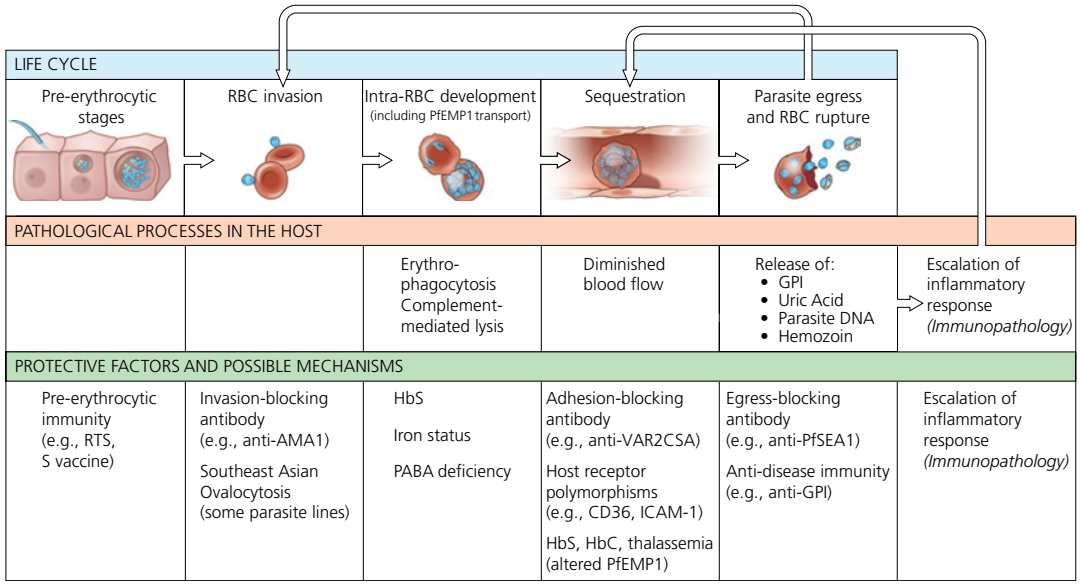

Figure 16.2 Pathological processes implicated in the pathogenesis of malarial disease are associated with specific phases of parasite development. Specific factors that reduce the risk of severe malaria are thought to affect these pathological processes or to prevent the initial infection that can result in severe malaria. These factors often form the conceptual basis for adjunctive therapies to reduce mortality after severe malaria develops or for vaccines to prevent the development of severe malaria. (*See insert for color representation of this figure*.)

interventions that prevent severe disease specifically, rather than those that limit parasite burden or mild disease.

In utero exposure

A child's *in utero* experience with maternal malaria also influences his or her risk of infection and disease during early life. In Cameroon, infants born to mothers with pregnancy malaria had higher susceptibility to infection than other children (Le Hesran 1997). Offspring of infected multigravidas but not infected primigravidas had a higher risk of parasitemia in Tanzania (Mutabingwa 2005) and of clinical malaria in Gabon (Schwarz 2008). Placental malaria may also predispose offspring to severe malaria: In Tanzania, we have observed that severe malaria risk is greater in offspring of infected multigravidas (Goncalves 2014).

The mechanism by which placental malaria increases susceptibility to infection, clinical malaria and severe malaria is not understood. Offspring of women with placental malaria may have lower levels of antibodies against malaria-specific antigens during infancy (Bonner 2005). Kenyan children who are born to infected women but fail to develop cellular responses to malaria blood-stage antigens have a higher risk of malaria infection during early life (Malhotra 2009). *In utero* exposure might also have a general effect on the immune response, since Beninese children born to mothers with placental malaria also have an increased risk of nonmalarial infections (Rachas 2012). In our studies, Tanzanian offspring born to infected multigravidas experience higher parasite density during their infections (Goncalves 2014), which might contribute to their increased risk of severe malaria.

Age

In areas of stable malaria transmission, age is often used as a proxy for immunity: Immunity to severe malaria is acquired at a young age, immunity that reduces clinical malaria risk is acquired later, and immunity that controls parasitemia is acquired even later. Within nonexposed populations, a very different pattern emerges, as observed in several studies of transmigrant populations (see also the section on Clinical spectrum and mechanisms of disease). Among Indonesian transmigrants new to a malaria transmission zone, adults are more likely than children to develop severe disease following primary exposure to *P. falciparum* (Baird 1998). Despite this initial susceptibility, adults acquire immunity to malaria faster than children: One year after entering a transmission zone, adults have lower prevalence of malaria infection (Baird 1993) and lower parasite densities during infections (Andersen 1997).

One explanation for increased severe malaria in adults could be that changes in the immune system might predispose to disease. For example, adrenal and gonadal hormone levels change with age, and some such as dehydroepiandrosterone (DHEA) have been shown to have immune modulatory function (Hazeldine 2010). Adrenarche has been related to acquired immunity to malaria and might therefore explain the rapidity with which adult transmigrants become resistant. In Kenya, male adolescents with higher levels of dehydroepiandrosterone sulfate (DHEAS) had lower parasite densities, even after adjusting for age (Kurtis 2001). Similarly, adolescent girls who had higher DHEAS levels had lower parasite density and higher hemoglobin levels, after adjustment for age (Leenstra 2003).

Iron status

Host iron has been related to several malaria outcomes (Gwamaka 2012; Kabyemela 2008). Iron-deficient Somali nomads randomized to receive iron supplementation for 30 days experienced activation of preexisting malaria (Murray 1978). The effect of iron on malaria risk is particularly problematic because the same populations that most need supplementation are also those at greatest risk for malaria: pregnant women and children. A large randomized trial in Pemba, Tanzania, was terminated early when children who received iron and folic acid supplementation experienced a statistically significant increase in risk of malaria infection and death (Ojukwu 2009; Sazawal 2006). Although some other trials failed to find a similar association of iron supplementation and malaria risk, these studies typically incorporated intensive malaria surveillance, prevention, and/or treatment that might have ameliorated the effect of iron to increase malaria risk (Desai 2003).

Naturally occurring differences in iron status may also influence malaria risk. On the coast of Kenya, iron deficiency reduced the incidence of mild clinical malaria by 30% among children younger than 8 years (Nyakeriga 2004). In a Tanzanian birth cohort, iron deficiency reduced parasitemia (23%) and severe malaria (38%) to roughly the same degree that it reduced mild clinical malaria risk in Kenya, and importantly also reduced all-cause mortality in an area of intense malaria transmission (Gwamaka 2012).

Iron status demonstrates a similar relationship in pregnant women (reviewed in (Friedman 2009)). In Tanzania, iron deficiency was associated with a significantly reduced risk of placental malaria in primigravidas and secundigravidas, but not multigravidas whose immunity may have mitigated the increased susceptibility due to increased iron stores (Kabyemela 2008). In Malawi, iron-replete pregnant women of all parities were significantly more likely to have placental malaria at delivery (Senga 2011). Maternal iron supplementation also enhances the risk of placental infections (reviewed in Friedman 2009).

Iron deficiency or supplementation might influence malaria risk through a number of mechanisms involving either the parasite or the host. For example, chelation of intraerythrocytic iron

reduces parasite growth (Hershko and Peto 1988), and chelation of iron increases cellular nitric oxide (NO) production and parasite killing *in vitro* (Fritsche 2001). Iron suppresses inducible NO synthase (iNOS) activity, and therefore iron deficiency might increase iNOS-mediated killing of blood-stage (Fritsche 2001) or liver-stage parasites (Klotz 1995).

In mice, liver-stage parasite development is inhibited by hepcidin, the iron regulatory hormone whose synthesis is upregulated during blood-stage infection (Portugal 2011). Both *P. falciparum* and *P. vivax* infections increase hepcidin levels (de Mast 2010). Whether hepcidin upregulation has an impact on the epidemiology of infection and of clinical disease in human populations needs to be further investigated.

Co-infections

In malaria-endemic areas, children are exposed to many pathogens and often present with co-infections. Co-infecting pathogens might interact with the malaria parasite to influence disease severity and acquisition of immunity to either agent. In this section we highlight the major co-infections that have been associated with *P. falciparum* malaria and any evidence that they modify malaria severity and immunity.

Invasive bacteremia

Invasive bacteremia is an important cause of death on the African continent. The overlapping spatial distributions of malaria and invasive bacteremia as well as similar temporal trends indicate that these two conditions might be associated. Malaria might increase the risk of invasive bacterial infection (Brent 2006), especially of non-typhi salmonellosis (NTS), a common invasive bacteremia in sub-Saharan Africa. In Kenya, sickle cell trait was associated with protection against invasive bacteremia (Scott 2011), probably due to its protective effect against malaria. In Tanzania, a rapid decline in malaria incidence coincided with a rapid decline in all-cause bacteremia among children, especially NTS (Mtove 2011). Among patients with diagnosed severe malaria, invasive bacteremia has been associated with increased disease mortality in some (Berkley 1999) but not all studies (Bronzan 2007). severe malarial anemia may have a specific relationship to invasive NTS. In mouse models, hemolysis due to malaria increases susceptibility to NTS infection (Roux 2010), as does induction of heme oxygenase 1, which impairs the granulocyte oxidative burst and therefore NTS killing (Cunnington 2012).

HIV infection

The reported relationship between malaria and HIV has varied widely between studies. Among children living in areas with stable transmission, HIV has been associated with no change in malaria susceptibility (Greenberg 1991), decreased susceptibility to parasitemia (Bebell 2007), and increased susceptibility to symptomatic malaria among young children (Ezeamama 2012) or to severe malaria among HIV^+ children and adolescents younger than 15 years (Hendriksen 2012).

In adults, HIV infection has been more clearly associated with malaria outcomes, possibly owing to the impact of HIV to impair acquired immunity. In Uganda, HIV infection in adults is associated with more frequent clinical malaria episodes and parasitemia (Whitworth 2000). In Malawi, HIV seropositivity is associated with increased risk of malaria infection (Patnaik 2005). HIV-infected adults with CD4 count less than 200 cells/mm^3 have higher incidence of clinical malaria compared to those with CD4 count greater than 500 cells/mm^3 (Laufer 2006), and HIV-1 infection is a risk factor for severe malaria among Zambian adults (Chalwe 2009).

HIV also increases the risk of placental malaria. In Kenya, the prevalence of placental malaria is higher among HIV seropositive versus seronegative gravidas (van Eijk 2003), although immunity increases with parity in both groups (ter Kuile 2004). Levels of antibody to placental IE surface

proteins may be higher in HIV^- versus HIV^+ women (Mount 2004), supporting the notion that HIV increases susceptibility through impaired immunity to malaria.

Just as HIV increases malaria risk, so too might malaria increase HIV risk, although this possibility requires further study. Viral loads increase and $CD4^+$ levels decrease during acute malaria episodes (Kublin 2005; Van Geertruyden 2006). Repeated malaria episodes have the potential to accelerate HIV disease progression (Mermin 2006). Based on a mathematical model, 8500 excess HIV infections and 980,000 excess malaria episodes were estimated to have occurred between 1980 and 2007 in an area of Kenya with an adult population of roughly 200,000 (Abu-Raddad 2006). However, viral loads return to baseline within two months of malaria infection, and HIV disease progression may be similar between malarious and nonmalarious areas (Kublin 2005). Similar uncertainty surrounds the effect of placental malaria on risk of mother-to-child transmission of HIV, with studies showing increased risk (Brahmbhatt 2008), decreased risk (Ayisi 2004), or no effect (Msamanga 2009).

Helminth infections

Helminth infections are highly prevalent throughout sub-Saharan Africa, and children older than 1 year often harbor these parasites. Chronic helminth infection is associated with Th2 and regulatory immune responses and therefore might modulate inflammatory responses and malaria outcomes. However, the relationship of helminths to risks of malaria infection and disease remains unclear. *Ascaris lumbricoides* was protective against malaria in the Comoros islands of the Indian Ocean (Murray 1977) and was less common in patients with cerebral malaria versus uncomplicated malaria in Thailand (Nacher 2000). However, in Senegal, intestinal worms were associated with an increased number of malaria attacks (Spiegel 2003), and *Ascaris lumbricoides* infection increased risk of severe malaria (Le Hesran 2004). Hookworm, a major soil-transmitted helminth, might also exacerbate anemia caused by malaria (Brooker 2007).

Schistosomiasis has also had an inconsistent relationship with malaria risk in different studies. In Senegal, children infected with *Schistosoma mansoni*, particularly those with high parasite loads, suffered clinical malaria more commonly than uninfected children (Sokhna 2004). In Mali, malaria incidence was lower in children infected with *Schistosoma haematobium* versus other children, and this effect was age-dependent (Lyke 2005). Malian children with *S. haematobium* infection had higher baseline levels of IL-4, IL-6, IL-10, and IFN-γ (Lyke 2006) and higher levels of antimalarial antibodies (Diallo 2010), suggesting that immune responsiveness may have contributed to their malaria-resistant status.

Filariasis also modulates the immune response to falciparum infection and suppresses malaria antigen–specific IL-12p70/IFN-γ production *in vitro* (Metenou 2009). Filarial infection was associated with protection against anemia during the malaria transmission season, despite having no effect on clinical malaria incidence or parasite density (Dolo 2012).

A better understanding of malaria–helminth co-infections might provide insights on malaria immunopathology and immunity acquisition. However, the relationships observed between malaria and helminth infections have been complex and often contradictory. Animal models have similarly shown contrasting results, which might be related to different parasites (type of helminth parasite and malaria strain) and hosts used in different models (reviewed in (Hartgers and Yazdanbakhsh 2006)).

Mixed malaria infections

Interactions between malaria parasite species might influence their population-level distributions as well as the course of infection and disease. *P. malariae* and *P. falciparum* parasitemias may increase concomitantly. Prior *P. malariae* infection but not *P. vivax* infection prevents fever related to *P. falciparum* infection and is associated with lower falciparum parasite burden (Collins and Jeffery 1999).

These patterns suggest that cross-reactive immune responses act simultaneously against *P. malariae* and *P. falciparum* (Collins and Jeffery 1999).

P. vivax and *P. falciparum* show the opposite pattern of interaction. In experimental human infections, *P. vivax* density only increases after *P. falciparum* parasitemia resolves (Boyd MF 1937). Mixed infections with *P. vivax* and *P. falciparum* occur less often than expected by chance when determined by bloodsmear patency (Maitland 1996). Conversely, the incidence of mixed infections appears random when measured by more-sensitive PCR techniques (Mehlotra 2002), suggesting that the interaction between malaria parasite species primarily influences parasite density rather than infection risk.

These epidemiological findings correspond to patterns observed in experimental infections, and suggest cross-species regulation of parasite growth. In an intensive longitudinal study in Papua New Guinea, different parasite species often did not coincide on their days of peak parasite density. This pattern might be explained by species-transcending regulatory mechanisms such as innate immunity (Bruce and Day 2003), but the varying interactions among species suggests that more than one mechanism might be involved.

Mixed infections might also influence malaria severity, although the pattern has not been consistent among study sites. In Nigeria, the risk of anemia was higher in multiple-species infections compared to single-species infections (May 2000). Conversely, mixed infections were associated with higher hemoglobin levels in Malawi (Bruce 2008).

Parasite virulence

Proinflammatory cytokines play an important role in the pathology associated with malaria infection. Several parasite factors may stimulate host proinflammatory immune responses, including hemozoin, parasite DNA (carried by hemozoin), GPI, often described as the malaria toxin, and uric acid generated from parasite-released precursors. Separately, the localization and burden of sequestered parasites has been associated with severe malaria in a number of studies, suggesting that parasite adhesion may be a key factor in disease outcomes. In this section, we review the evidence that these factors might contribute to severe malaria pathogenesis.

Hemozoin

Malaria parasites digest hemoglobin, leading to the production of toxic heme metabolites. Through a detoxification process, the parasite transforms heme into an insoluble crystal called hemozoin, which persists in the food vacuoles of the parasite. During schizogony, hemozoin is released into the circulation and is then captured by cells of the reticuloendothelial system. Purified hemozoin from *P. falciparum* induces cytokine, chemokine, and NO production by macrophages (Sherry 1995). Hemozoin induces IL-1β production through a pathway dependent on the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome (Shio 2009), enhancing host inflammation. Cerebral malaria fails to develop in Nalp3-deficient mice, highlighting the potential importance of hemozoin-related inflammasome activation in disease (Dostert 2009). Whereas hemozoin induction of innate responses may beneficially contribute to acquired immunity, the proportion of hemozoin-containing macrophages and free plasma hemozoin has been associated with severe malarial anemia (Awandare 2007; Casals-Pascual 2006), possibly due to hemozoin-induced modulation of cytokine and chemokines (reviewed in (Perkins 2011)).

Parasite DNA

Although the inflammatory response to hemozoin has been imputed to TLR9, an endosomal receptor (Coban 2005), the activity of hemozoin on TLR9 disappears after nuclease treatment, suggesting that it acts by carrying malaria parasite DNA into an intracellular compartment where it

is sensed by TLR9 (Parroche 2007). Although the malaria genome is AT-rich, classic CpG motifs as well as certain immunostimulatory AT motifs have been identified. TLR9 polymorphisms influence the risk of symptomatic malaria in Ghana, consistent with a role for this pathway in immunopathogenesis (Omar 2012). Parasite DNA might also influence the innate immune response to malaria via a receptor independent of TLR9 (Sharma 2011).

Glycosylphosphatidylinositol

GPI anchors some surface proteins to the cell membrane, including many *Plasmodium* proteins. Although GPI is present in all eukaryotic cells, GPI moieties present in mammalian cells and in protozoa differ structurally (distinct linked residues) and quantitatively (lower number of GPI copies on mammalian cells).

GPI of *P. falciparum* blood-stage parasites might trigger systemic inflammatory cascades that lead to pathological changes and disease (Schofield and Hackett 1993). Parasite-derived GPI induces expression of cell adhesion molecules (ICAM-1, VCAM-1, E-selectin) that support parasite cytoadherence (Schofield 1996). Residents of endemic areas develop anti-GPI antibodies. Levels of antibodies are related to age: Adults, who are normally protected against clinical malaria, have higher levels of antibodies, and young children have much lower levels. Anti-GPI antibody responses have been associated with protection against malarial anemia and fever (Naik 2000). GPI regulates NFkB/rel-dependent gene expression of TNF-α, and antibodies against GPI block TNF production induced by *P. falciparum* extracts (Tachado 1997). Naturally acquired anti-GPI antibodies partially neutralize macrophage activation by *P. falciparum* schizont extract, suggesting that components other than GPI also contribute to macrophage activation (de Souza 2010).

GPI might be important in the pathogenesis of severe malaria as well as uncomplicated malaria. In Senegal, anti-GPI IgGs were significantly higher in individuals with mild malaria compared to patients hospitalized with cerebral malaria (Perraut 2005). Mice immunized with synthetic GPI are protected against severe manifestations of malaria infection (murine cerebral malaria, pulmonary edema, and acidosis) despite having similar parasite densities during infection (Schofield 2002). In pregnant women, anti-GPI antibody levels increase during malaria infection but are not associated with TNF levels or pregnancy outcomes (Suguitan 2004).

Uric acid

Plasmodium parasites are dependent on an external purine source, resulting in the accumulation of hypoxanthine and xanthine in IE (Orengo 2008). Hypoxanthine released during schizont rupture is degraded by xanthine oxidoreductase (XO), leading to formation of uric acid. Uric acid stimulates peripheral blood mononuclear cells to produce inflammatory cytokines TNF-α IL-1β, and IL-6; conversely, XO inhibitors reduce the levels of cytokine secreted by PBMC stimulated with IE (Orengo 2009). The pathway by which uric acid stimulates the production of TNF remains to be determined (Orengo 2009).

An early study using the rodent parasite *P. berghei* revealed a sharp increase in XO levels 4 days after infection, similar to that observed with bacterial infections (Tubaro 1980). Treatment with the XO inhibitor allopurinol resulted in increased mortality in a dose-dependent manner (Tubaro 1980).

In contrast to the mouse studies, allopurinol accelerated the clearance of parasites and fever among malaria-infected patients who were also receiving quinine, possibly due to its effects on purine biosynthesis in the parasite (Sarma 1998). In Mali, uric acid levels correlated with parasite density in children with uncomplicated malaria, and they further increased in children with severe malaria (Lopera-Mesa 2012). Uric acid levels correlated with levels of inflammatory mediators; both parasitemia and creatinine levels were related to uric acid levels, suggesting that hypoxanthine released from IE, as well as kidney function, might be contributing to the increase in uric acid (Lopera-Mesa 2012).

Parasite adhesion

Alphonse Laveran, who discovered the malaria parasite in Algeria in 1880, also related the severity of disease to the degree of parasite sequestration in vascular beds (Laveran 1882), including the accumulation of parasites in cerebral capillaries as a cause of neurologic manifestations. Autopsy studies have confirmed that cerebral malaria is associated with increased parasite burden in cerebral vessels (MacPherson 1985; Pongponratn 1991). These clinicopathological correlates have inspired speculation that parasites may be binding to a specific endothelial receptor in the brain. Over years of research, several receptors expressed by vascular endothelium have been shown to support IE binding *in vitro*, including CD36, thrombospondin, ICAM-1, PECAM-1, VCAM, E-selectin, P-selectin, CSA, gC1qR/HABP1 (gC1qR), EPCR, and possibly integrin molecule $\alpha_v\beta_3$ (Barnwell 1985; Berendt 1989; Biswas 2007; Fried and Duffy 1996; Ho 1998; Ockenhouse 1992; Roberts 1985; Siano 1998; Treutiger 1997; Turner 2013). The host receptors supporting parasite adhesion during severe malaria have yet to be conclusively defined, and the binding properties of severe malaria parasites remain a subject for further research (Table 16.2).

ICAM-1 has been proposed to mediate IE binding in the brain based on the following observations: TNF-α upregulates ICAM-1, E-selectin, and VCAM-1 expression on vascular endothelium (Turner 1994), and IE co-localizes with ICAM-1 in brain vessels (Ockenhouse 1992; Turner 1994). However, activation of ICAM-1 expression is not limited to brain endothelium, and other tissue beds co-express CD36 and ICAM-1. Most studies failed to associate ICAM-1–binding parasites with severe malaria (Table 16.2). Two studies reported increased IE binding to ICAM-1 during severe malaria cases and to CD36 during uncomplicated malaria (Ochola 2011; Turner 2013). A common ICAM-1 polymorphism present in African populations is not associated with reduced IE binding to ICAM-1 (Craig 2000) or reduced risk of severe malaria including cerebral malaria (Fry 2008). IE adhesion to human brain microvascular endothelial cells (HBMECs) is not mediated by CD36 or ICAM, suggesting that other unknown receptors might be involved in the process of cerebral cytoadhesion and sequestration (Avril 2012). In Tanzania, isolates from children with severe malaria bind EPCR (and ICAM-1) at higher levels, but EPCR was absent at the site of IE adhesion in the brains of Malawian children who died of cerebral malaria (Moxon 2013).

In Kenya, IE collected from children with asymptomatic malaria, nonsevere malaria, severe malarial anemia, or cerebral malaria were compared (Newbold 1997): All samples bound at high levels to CD36, and a large fraction of the isolates (80%) adhered to the receptor ICAM-1 as well; IEs bound to E-selectin and CD31 less commonly and at lower levels (Newbold 1997). However, binding of parasites to any of these receptors was not significantly related to severe malaria, and the roles of these receptors in specific clinical syndromes remains to be confirmed (Newbold 1997). Another study from the same site reported that IE collected from children with severe malaria tend to bind to multiple receptors (Heddini 2001).

In Thailand, IE binding to CD36 did not differ between cerebral malaria versus uncomplicated cases (Ockenhouse). Platelets have been implicated in sequestration and pathogenesis of cerebral malaria: Platelets accumulate to a higher degree in brain vessels of cerebral malaria cases compared to other fatal cases; CD36 is expressed on the surface of platelets and could hypothetically play a role in IE sequestration in brain blood vessels (Grau 2003).

Specific syndromes may result from multiple ligand–receptor interactions, and these could be positive or negative interactions. As an example of negative interaction, IE in the placenta binds to CSA and does not bind to the receptor CD36 (Fried and Duffy 1996). Alternatively, IE binding to multiple receptors might enhance binding avidity, as has been observed *in vitro* under static or

Table 16.2 SM and adhesion to specific endothelial receptors.

Receptor	Comparison groups	Findings	Reference
CD36	SMA vs UM CM vs UM	Significantly higher in UM No difference	Newbold 1997
	SM[1] vs UM SMA vs UM	Significantly higher in UM Significantly higher in UM	Rogerson 1999
	SM[2] vs UM	No difference	Mayor 2011
	SMA vs UM CM vs UM	Significantly higher in UM	Ochola 2011
ICAM-1	SM[3] vs UM	No difference	Ockenhouse 1991
	SMA vs UM CM vs UM	Significantly higher in UM No difference	Newbold 1997
	SM[1] vs UM CM vs UM	Significantly higher in UM No difference	Rogerson 1999
	SM[2] vs UM	No difference	Mayor 2011
	CM vs UM	Significantly higher in CM under flow conditions	Ochola 2011
	SM[4] vs UM	Significantly higher in SM	Turner 2013
VCAM	SMA vs UM CM vs UM	No difference	Newbold 1997
PECAM-1/CD31	SMA vs UM CM vs UM	No difference	Newbold 1997
E-selectin	SMA vs UM CM vs UM	No difference	Newbold 1997
gC1qR	Seizures vs UM	Significantly higher in seizure cases	Mayor 2011
Endothelial protein C receptor (EPCR)	SM[4] vs UM	Significantly higher in SM	Turner 2013
Multi-adhesion[5]	SM[6] vs UM	Significantly higher in SM	Heddini 2001
Multi-adhesion[5]	SMA vs UM	Significantly higher in SMA	Heddini 2001
Multi-adhesion[5]	CM vs UM	No difference	Heddini 2001

UM: Uncomplicated malaria
SMA: Severe malaria anemia
CM: CM
[1] SM: CM, SMA or both
[2] SM: prostration, acidosis and/or RD, severe anemia, seizures, CM, hypoglycemia. No differences were observed in comparisons between UM and individual SM syndromes
[3] SM: CM, acute renal and hepatic dysfunction
[4] SM: CM, SA
[5] Multi-adhesion: score based on binding to multiple receptors (Ig, sPECAM/CD31, PECAM/CD31, CD36, resetting and giant rosetting)
[6] SM: prostration or hyperparasitemia (>20%)
(Heddini 2001; Mayor 2011; Newbold 1997; Ochola 2011; Ockenhouse 1991; Rogerson 1999; Turner 2013).

flow conditions with cells co-expressing CD36 and ICAM-1 (Gray 2003; McCormick 1997). Some studies have found that clinical IE samples from severe malaria cases bind multiple receptors (Heddini 2001), but none have conclusively identified specific receptor combinations that characterize specific syndromes.

Parasite rosetting is an additional adhesion mechanism by which IEs bind to uninfected erythrocytes (David 1988). In some studies, rosetting parasites have been related to severe malaria: Rosette formation is more common among parasite samples collected from children with cerebral malaria compared with those with uncomplicated malaria (Treutiger 1992) or it achieves higher levels among parasite samples collected from individuals with severe anemia (Heddini 2001; Newbold 1997) or with any severe malaria syndrome (Doumbo 2009). Conversely, a number of studies found no association between rosetting and severe malaria, including cerebral malaria cases (al-Yaman 1995,Rogerson 1999). The variable relationship between rosetting and disease severity could result if severe malaria pathogenesis differs between populations, and rosetting is involved in some but not others of these pathogenic processes (Rowe 2009). Another possibility is that the genetic differences between populations, such as red cell properties, might variably affect rosetting and its relationship to disease (Rowe 2007).

Several factors confound the effort to relate adhesion receptors to specific syndromes, such as misclassification of cases and limitations of sampling methods. Even with strict adherence to diagnostic criteria, about 25% of clinically diagnosed cerebral malaria cases are incorrect by histopathological examination (Taylor 2004). Some children with uncomplicated malaria may be early in the process of developing severe malaria. Children with cerebral malaria or other severe malaria syndromes may have overlapping clinical manifestations (Maitland and Marsh 2004), and the peripheral blood samples therefore might contain IE associated with multiple syndromes. Similarly, peripheral blood parasites collected from children with severe malaria include an unknown fraction emanating from vascular beds that are not involved in the severe malaria process, such as parasites sequestered in dermal vasculature.

Surface proteins of infected erythrocytes

The variant IE surface protein called PfEMP1 has been implicated in adhesion to several endothelial receptors as well as in antigenic variation, and it is thus believed to play a key role in severe disease caused by *P. falciparum*. Each parasite expresses one PfEMP1 variant on the IE surface (Scherf 1998) but can switch to a different variant after invasion of a new erythrocyte (Roberts 1992). PfEMP1 variants are encoded by approximately 60 *var* genes per haploid genome of *P. falciparum* and display extensive variation within and between genomes. The extensive variation in *var* gene sequences may result from recombination during mitosis (Freitas-Junior 2000) as well as meiosis (Duffy 2001).

Sequence analysis of the 3D7 laboratory isolate genome classified *var* genes into three major groups (A, B, and C) and two intermediate groups (B/A and B/C) based on homology within the flanking regions of the coding sequence (Lavstsen 2003). The classification of *var* genes into these groups was similar in additional parasite isolates, suggesting that upstream regions are important in the evolution of the *var* gene family (Kraemer 2007). *Var* genes in groups B and C have greater sequence homology compared to *var* genes of group A, but for all the *var* genes, the degree of similarity is higher among *var* genes within the same group (Kraemer 2007). While 58 *var* genes in the 3D7 genome carry upsA, upsB, or upsC sequences, two *var* genes carry unique sequences, including the VAR2CSA gene that is associated with placental parasites. The ups types also correspond to other genetic features of PfEMP1, including domain architecture, chromosomal location, and Duffy-binding-like (DBL) 1 and cysteine-rich interdomain region (CIDR) sequence homology (Kraemer 2007).

The classification of *var* genes into several major groups is associated with adhesive function. For example, recombinant CIDR domains of group A do not bind CD36, unlike CIDR domains of other groups (Robinson 2003). Further comparative analysis of *var* gene sequences resulted in the identification of domain cassettes (DCs) (Rask 2010). Domain cassettes comprise two or more

consecutive domains shared between multiple *P. falciparum* genomes that are part of the same subclass described above (Salanti 2003).

Despite the enormous diversity of *var* gene sequences, a few PfEMP1 molecules are highly conserved between isolates from around the world (Fried and Duffy 2002; Jensen 2004). Some conserved PfEMP1 forms have been implicated as surface proteins of the IE that cause pregnancy malaria (VAR2CSA) (Salanti 2003) or severe malaria (Jensen 2004). Selection of 3D7 parasites with sera from semi-immune children from East Africa or West Africa is accompanied by upregulation of transcription of two group A genes, prompting speculation that the corresponding PfEMP1 forms are targeted by protective antibody (Jensen 2004). Over the course of chronic infections, IEs modify their PfEMP1 expression. This may reflect selection against successively expressed variant surface proteins by antibodies as they are acquired, as has been observed in rhesus monkeys infected with *P. knowlesi* (Brown 1973; Brown and Brown 1965), which expresses a different family of variant surface antigen called SICAvar.

The dominance of *P. falciparum* parasites expressing specific PfEMP1 forms might be related to their higher affinity or enhanced sequestration and therefore to their higher growth rate in individuals without preexisting immunity to these PfEMP1 variants. Antibody selection against these high-affinity PfEMP1s over the course of chronic infection might yield PfEMP1 proteins that bind with lower affinity and therefore with potentially less virulence. Thus, the shift toward other parasite (and PfEMP1) forms might occur as antibody develops against the PfEMP1 variants expressed by severe malaria parasites and counteracts their selective growth advantage.

Several studies attempted to correlate transcription of *var* gene groups with disease. In Brazil, IE from severe malaria cases are enriched for *var* genes belonging to groups A and B/A (Kirchgatter and Portillo Hdel 2002). In Kenya, some but not all parasite samples collected from children with severe malaria preferentially expressed group A *var* genes (Bull 2005). IE from Malian children with cerebral malaria preferentially expressed group A and B/A *var* genes (Kyriacou 2006). Conversely, IE collected from children in Papua New Guinea with mild and severe malaria were more likely to express group B *var* genes, but those from asymptomatic children were more likely to express group C *var* genes (Kaestli 2006). In Kenya and Papua New Guinea, IEs that form rosettes (bind to uninfected erythrocytes) preferentially express group A *var* gene transcripts. As noted above, selection of laboratory isolates on sera from semi-immune children induces the expression of group A *var* genes (Jensen 2004).

The difficulty in correlating discrete *var* genes with clinical syndromes is similar to the complexity of relating IE adhesion phenotype with disease (see the section on parasite adhesion). Severe malaria encompasses a number of different clinical syndromes, and each might be associated with the expression of a subgroup of *var* genes. For example, *var* gene domain cassettes 8 and 13 (DC8, DC13) are upregulated in parasites that bind to human brain microvascular endothelial cells (HBMECs) (Avril 2012; Claessens 2012), and parasites from children with severe malaria preferentially express DC8 and DC13 (Lavstsen 2012). Further study is needed to determine whether these domain cassettes are related to a specific syndrome, such as cerebral malaria.

Although much attention has been given to PfEMP1 protein-expression profiles, the possibility remains that other surface antigens might contribute to severe malaria pathogenesis. Additional variant antigens belonging to the gene families RIFIN, STEVOR, and PfMC-2M (*P. falciparum* Maurer's clefts 2 transmembrane) have been described (Cheng 1998; Kyes 1999; Sam-Yellowe 2004). Several studies have demonstrated that proteins expressed by these gene families localize to the Maurer's clefts that form within red cell cytosol, but only a few studies have suggested that RIFIN and STEVOR proteins may be displayed on the IE outer membrane surface (Bachmann 2012, Kyes 1999; McRobert 2004; Sam-Yellowe 2004). To date, conserved antigens encoded by single-copy genes have not been detected on the IE surface.

Conclusions

The rapid acquisition of immunity to severe malaria in endemic areas cannot be explained by immunity that controls parasite density or that reduces mild malaria, but it could be explained by immunity to parasites that display limited antigenic diversity, and several lines of evidence support this notion. Children with clinical malaria carry IE to which they lack pre-existing antibodies (Bull 1998), suggesting that immunity is variant specific. Sera from children who are resistant to symptomatic malaria can be used to select parasite forms *in vitro* that express distinct forms of PfEMP1, the major variant antigen on the IE surface (Jensen 2004). Children with severe malaria carry IEs that are commonly recognized by sera from the community (Bull 2000), suggesting that these IEs express surface proteins with conserved features. If so, the IE causing severe malaria might be targeted by protective antibodies as a child grows older and becomes resistant to severe malaria (Bull 2000).

Hypothetically, IEs expressing surface proteins that bind with higher affinity to endothelium might enjoy enhanced sequestration and therefore higher growth rates, compared to other IEs. Enhanced growth would allow IEs expressing these high-affinity surface proteins to dominate infections of nonimmune hosts. Severe disease may be related to IE surface proteins that bind with highest affinity to specific receptors in vascular beds related to disease, such as the brain in cerebral malaria, leading to increased local parasite burden. The IE surface proteins with highest affinity to these specific receptors might also be functionally constrained in their degree of antigenic variation, which would explain why children might only suffer one or two episodes of severe malaria on average before acquiring protective immunity. The advantage for parasites that express these surface proteins will diminish as the host acquires specific antibody that blocks their interaction with receptors or that facilitates opsonophagocytosis of IEs expressing these surface proteins. Antibody selection might then favor parasites expressing PfEMP1 proteins that bind with lower affinity and therefore with potentially less virulence.

Despite the importance of parasite burden to the development of severe disease, individuals often harbor high parasite biomass without developing life-threatening symptoms. The vascular beds that support sequestration may vary between infections, and this could partly explain the different presentations of malaria. Further, although the sequestered parasite biomass may play an essential role in severe malaria pathogenesis, immunopathology might also contribute to the cascade of events leading to serious malaria morbidity. Increased parasite biomass may increase the likelihood of specific parasite–host interactions that catalyze inflammation, endothelial dysfunction, and the cascade of events that culminate in severe disease. In addition, inherent host differences in immune responsiveness, such as those caused by *in utero* exposures or by genetic polymorphisms, might explain why some children are intrinsically resistant to severe disease.

The IE surface proteins that optimally support adhesion and parasite growth might change over time in the host, owing to the different affinities of specific ligand–receptor interactions, the accumulating repertoire of antibodies against IE surface proteins, and possibly an evolving set of endothelial receptors in deep vascular beds of the growing child. In this way, the changing presentation of severe malaria with age may be explained by the diversity of parasite surface proteins, their interactions with a varying repertoire of host endothelial receptors in different vascular beds, and the host immune response. This model suggests that children might be protected from severe malaria by vaccines that accelerate the acquisition of immunity against specific PfEMP1 and other surface proteins expressed by severe malaria parasites.

Once the cascade of events leading to severe malaria has been initiated, a number of coinciding phenomena may separately contribute to poor outcomes. Inflammation, vascular occlusion, endothelial dysfunction and leakage, and metabolic acidosis might each contribute separately and

together to tissue damage and disease pathogenesis. Although each or all of these might be important contributors to development of disease, reversing these processes to prevent mortality might be difficult once an individual develops severe malaria, as indicated by the lack of efficacy of most adjunctive treatments that target these processes. Notably, albumin infusion might reduce mortality during severe malaria, perhaps by circulatory support and/or reversing acidosis.

The optimal solution to severe malaria will be a vaccine that prevents infections or that eradicates malaria. Before achieving these lofty goals, a vaccine that prevents severe malaria without reducing infection might be feasible, as indicated by epidemiologic studies in areas of stable malaria transmission. Such a vaccine becomes even more important in areas that are progressing toward elimination, because their populations are progressively losing immunity and increasing their risk of severe malaria during infection at all ages. An important consideration will be whether independent vaccines may be required for each severe malaria syndrome, or whether specific antigens may be shared between parasites causing different syndromes and therefore be tractable to a single vaccine. Future studies should confirm whether immunity that arises after one severe syndrome might confer resistance to all severe syndromes, as has been suggested by cohort studies (Goncalves 2014), or only to the syndrome that first appeared in the child.

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CHAPTER 17
Host genetics

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As described in detail by a number of authors throughout this book, malaria has been a preeminent cause of child mortality in tropical and subtropical regions of the world throughout much of the last 5000 years. Through most of that period the biology of the disease was poorly understood and no effective methods for prevention or treatment were known. Nevertheless, malaria was not universally fatal: even though complete immunity is rarely reached, the majority of children exposed to malaria survive. The factors determining the outcome of each new exposure are complex, but they probably relate in part to parasite genetic factors, levels of preexisting immunity, and the nutrition and health status of the host (Greenwood 1991), areas that have been covered in chapters 15 and 16 of this book. However, it is also clear that to a considerable degree, disease outcome is also influenced by host genetic factors – the focus of the current chapter.

What evidence is there that the risk of malaria is genetically determined?

In a classic early study of malaria heritability, Abel and colleagues used segregation analysis to show that genetic factors had a major influence on peripheral blood film malaria parasite densities among 285 members of 42 nuclear families living in southern Cameroon (Abel 1992). Subjects were sampled serially and data recorded on a range of epidemiological exposures. The authors found evidence for the presence of a recessive major genetic effect on peripheral parasite density: 23% of the population were predisposed to high parasite densities and 77% were resistant.

Similar results were reported from a series of subsequent studies conducted in different populations by the same group. First, Garcia and colleagues conducted a study of similar design in a second population in southern Cameroon consisting of 44 nuclear families of a different ethnic background to that included in the earlier study (Garcia 1998a). Although their findings were also compatible with the existence of a complex genetic trait controlling malaria parasite densities, the results were not consistent with the autosomal recessive model suggested by the initial study. Second, Cot and colleagues found evidence consistent with a major genetic effect on parasite densities among a cohort of 570 pregnant women in Burkina Faso (Cot 1993). After adjusting for significant confounders including area of residence and parity, parasite densities fell within a bimodal distribution, suggesting that around 5% of subjects were predisposed to high-density infections, an observation in keeping with a major genetic effect.

Advances in Malaria Research, First Edition. Edited by Deepak Gaur, Chetan E. Chitnis, and Virander S. Chauhan.

Although this series of studies supported a role for genetic factors in the control of malaria parasite densities, the fact that genetic factors are important in the control of clinically relevant outcomes, such as febrile episodes or severe disease, is supported by studies conducted by a number of other investigators. One of the first studies to address this question in humans was conducted by Jepson and colleagues in the Gambia (Jepson 1995). They approached this issue by comparing the incidence of clinical malaria in pairs of twin children within families who therefore shared identical environmental exposure to malaria. Twins were classified as monozygotic (children derived from a single zygote who are therefore genetically identical) or dizygotic (children derived from two separate zygotes who therefore share on average 50% of their genes) and were monitored for clinical malaria episodes throughout a full transmission season. The authors found that both members of a monozygotic pair of twins were less likely to carry parasites asymptomatically than both members of a dizygotic pair. This indicated that parasitemia *per se* was not under strong genetic control in their study population. However, monozygotic twins were more often concordant with regard to episodes of febrile malaria than were dizygotic twins, suggesting that this outcome was under some degree of genetic control.

The degree to which genetic factors influence the incidence of various forms of clinical malaria has been addressed by a number of investigators using pedigree-based genetic variance component analysis of cohort studies investigating a range of clinical outcomes. In a study of almost 2000 subjects of all ages conducted in Sri Lanka, Mackinnon and colleagues found that overall, genetic factors were responsible for roughly 50% of the variation in the risk of clinically significant malaria episodes (Mackinnon 2000). Interestingly, whereas genetic effects appeared to modify the risk of both *Plasmodium falciparum* and *Plasmodium vivax* malaria, a number of differences were apparent. Genetic effects appeared to modulate both the clinical course of *P. falciparum* episodes and of parasite densities within these episodes, but they appeared to determine more absolutely whether or not a subject became infected with *P. vivax* parasites. In a subsequent study conducted using the same approach in a population of children living in Kilifi on the coast of Kenya, Mackinnon and colleagues found that genetic factors explained approximately one quarter of the variability in the risk of uncomplicated episodes of clinical *P. falciparum* malaria and almost one third of the variability in the risk of more-severe episodes resulting in admission to hospital (Mackinnon 2005).

Subsequently, other investigators have made essentially similar observations in Thailand, where the contribution of genetic factors to the variability in the incidence of uncomplicated *P. falciparum* and *P. vivax* malaria infections in a large cohort of mixed ages was found to be 10% and 19%, respectively (Phimpraphi 2008), and in Senegal, where the genetic contribution to the incidence of *P. falciparum* malaria (28%) (Sakuntabhai 2008) was close to that seen in the Kenyan study (Mackinnon 2005). Finally, in a further analysis of the same study, Lawaly and colleagues noted a major contribution of genetic factors to the prevalence of gametocytes during asymptomatic *P. falciparum* infections, but they found no genetic contribution to gametocytemia during episodes of clinical disease (Lawaly 2010).

Taken together, these studies suggest that, as a whole, genetic factors play a substantial role in determining both the incidence and outcome of both *P. falciparum* and *P. vivax* malaria. Nevertheless, they tell us little about the specifics of which genes are involved.

Identifying the genes involved

In the sections that follow, we discuss a wide range of genes and gene regions for which evidence is available for a link to malaria susceptibility. In some cases, most notably polymorphisms affecting the structure or function of red blood cells such as the hemoglobin variant hemoglobin S (HbS) and the Duffy (Fy) blood group antigen, the evidence supporting an impact on malaria infections is

Table 17.1 Approaches to providing evidence for protective association between specific genetic factors and malaria.

Supportive evidence	Example	References
Correlation between allele frequency and malaria	α-Thalassemia	Flint 1998
	Sickle hemoglobin	Piel 2010
	DARC-negativity	Howes 2011
Molecular-genetic studies showing evidence for recent selection	G6PD-deficiency	Tishkoff 2001
Laboratory evidence of biological effect	G6PD-deficiency, sickle hemoglobin	Ayi 2004
Animal models	Sickle hemoglobin	Ferreira 2011
Epidemiological studies under natural exposure		
Reduced parasite prevalence or density	DARC-negativity	Kasehagen 2007
Cohort studies of malaria incidence	α-Thalassemia	Wambua 2006a
Case-control studies	Ovalocytosis	Genton 1995
Twin studies	HLA-B	Jepson 1997
Ethnic differences in malaria susceptibility	Immune regulatory genes	Torcia 2008
Experimental challenge studies	DARC-negativity	Miller 1976

overwhelming; in others, the evidence is much more tenuous, in some cases being limited to biological plausibility or to a single observational study. As we will see, a number of approaches have been taken to the identification of specific genes and gene regions that affect various phenotypes of malaria and to the subsequent investigation of the mechanisms through which they operate. Examples include observed correlations between the spatial distribution of candidate genes and that of malaria at local, regional, or global scales (Siniscalco 1961; Flint 1986; Piel 2010; Howes 2011); the use of family-based linkage studies (Garcia 1998b; Rihet 1998; Flori 2003a; Flori 2003b); the study of ethnic differences in malaria susceptibility (Modiano 1991; Modiano 1996); genetic studies linking the time-depth of selection for genes to that of human malaria (Tishkoff 2001); and a range of epidemiological approaches, traditionally including case-control and cohort studies of candidate genes and, more recently, newer approaches such as genome-wide and gene-expression studies (Griffiths 2005; Timmann 2007; Jallow 2009; Timmann 2012). Table 17.1 summarizes some of the approaches that have been taken to providing evidence for associations with specific genes.

Why is genetic resistance important?

The main driving forces behind the hunt for malaria resistance genes are the hope that learning how such genes affect malaria risk will tell us something more about the biology of malaria in humans and the dream that this will translate into new ways of preventing or treating the disease. Unfortunately, there are relatively few examples of ways either these hopes or these dreams have become a reality. Nevertheless, there is one: The work by Miller and colleagues, who first made the link between the absence of *P. vivax* malaria in Africa and the wide distribution of the

Duffy-negative Fy(a-b-) blood group (Miller 1975) to show that the Fy antigens were necessary for the invasion of red blood cells by the *P. vivax* parasite (Miller 1976), has since led to the development of a *P. vivax* vaccine that is now in clinical trials (Schwartz 2012), a story that will be further elaborated in the following section.

The outcome has been far less clear-cut in relation to the majority of other examples. The reasons for this will be discussed, but in general they reflect the extreme complexity of human malaria, a disease that varies in its clinical expression both temporally and spatially on account of diverse factors including transmission intensity, vector type, parasite-specific variability, host immunity, and the availability of care. Nevertheless, the study of genetic resistance factors has helped us to learn a number of important lessons, including the dissection of the main invasion pathways of malaria parasites into host red cells. Moreover, a better understanding of the role of genetic modifiers is important for other reasons, including how they affect the interpretation of clinical trials of drugs and vaccines (Parikh 2008).

Genetic polymorphisms of the red blood cell

As a group, polymorphisms that affect various aspects of red blood cell structure and function provide some of the clearest examples of genetic factors that influence the clinical course of human malaria infections.

Red cell blood group antigens

A wealth of evidence suggests a role for malaria in the evolution of a number of antigens expressed on the surface of red blood cells including Duffy, ABO, Knops, and a number of others.

Duffy antigen receptor for chemokines (DARC)

The Duffy (Fy) blood group antigens were first recognized more than 60 years ago in a patient of this name who had been in receipt of multiple blood transfusions (Cutbush 1950). It has been shown that the Fy antigens bind a wide range of pro-inflammatory chemokines, suggesting that they may play a role in modulating their concentrations in plasma (Fukuma 2003), an observation that has led to a change in the common nomenclature to Duffy antigen receptor for chemokines (DARC).

The genetic basis for DARC derives from two co-dominant alleles, *FY*A* and *FY*B* on chromosome 1 (Donahue 1968), which respectively encode the Fy^a and Fy^b blood group antigens. Mutations within the the *FY* gene have given rise to the null or erythrocyte silent (ES) phenotypes of the *FY*A* and *FY*B* alleles to result in four major Fy phenotypes: Fy(a+b+), Fy(a+b–), Fy(a–b+) and Fy(a–b–) (Langhi 2006). It has long been known that in contrast to the rest of the world, where most people express either one or both of the *FY*A* or *FY*B* alleles, the vast majority of subjects within sub-Saharan Africa are of the Fy(a–b–) (DARC-negative) phenotype (Sanger 1955; Cavalli-Sforza 1994). In a recent analysis of available data assembled from published and unpublished surveys conducted since 1950, Howes and colleagues confirmed that the allele frequency for the $FY*B^{ES}$ phenotype exceeds 90% across 30 countries within the region, and frequencies reach 100% in many parts of West, Central, and East Africa (Howes 2011).

DARC and malaria

The link between the DARC-negative phenotype and resistance to *P. vivax* infection was first suspected in the 1970s (de Carvalho 2011). Miller and colleagues noted that while the simian malaria parasite *Plasmodium knowlesi* was able to infect DARC-positive red blood cells, DARC-negative red blood cells were resistant to invasion (Miller 1975). Noting that the DARC-negative

phenotype occurred at high frequency in West Africa, where the population were known to be resistant to *P. vivax* infections, they speculated that DARC might also be the receptor for *P. vivax* invasion into red blood cells (Miller 1976). In a subsequent experiment, the same group showed that among 11 volunteers of various DARC phenotypes, only those expressing either the Fy^a or Fy^b antigens became infected with *P. vivax* on artificial challenge using *P. vivax*–infected mosquitoes, while 5 DARC-negative subjects were completely refractive to *P. vivax* infection (Miller 1976).

In the years that followed, the central role of DARC in *P. vivax* invasion has been elucidated through a range of studies by a number of groups, summarized by James Beeson and colleagues (Beeson 2007). We now know that invasion involves a complicated process comprising multiple steps that, ultimately, is critically dependent on a specific molecular interaction between DARC and the *P. vivax* Duffy-binding protein (PvDBP), a molecule that is secreted from the micronemes of the *P. vivax* merozoite (Chitnis 2008). Studies using *P. knowlesi*, a parasite that can be maintained for experimental purposes in Rhesus monkeys, have shown that although initial interaction and reorientation of *P. knowlesi* merozoites occurs normally in DARC-negative red cells, the junction between the merozoite and red blood cell membrane does not subsequently form, and invasion is aborted at this step (Miller 1979). Invasion is similarly aborted in *P. knowlesi* merozoites derived from a line in which the homologue of PvDBP, PkDBP, has been knocked out (Singh 2005). Further work has shown that, more specifically, invasion depends on an interaction between highly conserved sequences within the PvRII region of PvDBP (VanBuskirk 2004; Hans 2005; Singh 2006) and a 35–amino acid sequence within the N-terminal extracellular region of the DARC molecule (Chitnis 1996).

DARC and *P. vivax* vaccines

The central importance of DARC negativity as a protective factor against clinical *P. vivax* infections is further supported by work conducted in the Pacific. In the late 1990s, a small number of individuals in Papua New Guinea (PNG) were identified who were heterozygous for a newly described $FY*A^{ES}$ allele (Zimmerman 1999). These individuals were shown to express levels of *FY*A* on their red blood cells that were half those measured in normal subjects (Zimmerman 1999) and to be significantly protected against *P. vivax* infections in terms of parasite prevalence at cross-sectional survey, clinical *P. vivax* episodes, and parasite densities during incident *P. vivax* infections (Kasehagen 2007).

Another study has addressed a question to which the answer has long been a mystery. King and colleagues (King 2011) investigated the binding of recombinant PvRII to human red cells of a range of different DARC phenotypes. They found that cells expressing Fy^a bound roughly half as much PvRII as cells expressing Fy^b, suggesting that *in vivo*, subjects expressing Fy^a might be less susceptible to clinical malaria than those expressing Fy^b. The authors went on to explore this hypothesis through an epidemiological study conducted in Brazil, and they confirmed a strong relationship between *FY* genotype and the incidence of clinical *P. vivax* malaria, the incidence being highest in *FY*B/FY*B* and lowest in $FY*A/FY*B^{ES}$ subjects (King 2011).

The importance of DARC as a receptor for *P. vivax* invasion has provided support for a vaccine-development strategy that targets the blockade of *P. vivax* invasion in DARC-positive individuals. This approach is supported by the observation that recombinant proteins based on PvRII can generate antibodies in rabbits and that both these and human antibodies of the same specificity, derived by affinity purification from *P. vivax*–exposed adults, inhibit the invasion of DARC-positive red blood cells by *P. vivax* parasites *in vitro* (Grimberg 2007). Moreover, in studies conducted under conditions of natural exposure, there is a strong negative correlation between the presence of naturally occurring antibodies that inhibit binding of PvRII to DARC and *P. vivax* infections (King 2008). Such observations suggest that a vaccine designed to generate antibodies that block the interaction between PvRII and DARC might be useful in preventing *P. vivax* infections in DARC-positive subjects.

One such vaccine, PvDBPII, an *Escherichia coli*–expressed recombinant protein consisting of the receptor binding domain of the *P. vivax* Duffy-binding protein (PvRII) is in preclinical development at the International Centre for Genetic Engineering and Biotechnology (ICGEB) in New Delhi (Schwartz 2012).

Other developments

Despite this generally good news story, data suggest that *P. vivax* parasites may be evolving to escape their dependence on DARC. In a study conducted in Western Kenya, Ryan and colleagues found parasites that behaved like *P. vivax* in both humans and mosquitoes, although insufficient material was available for molecular confirmation (Ryan 2006). However, *P. vivax* parasites have been identified in a small number of DARC-negative subjects in Brazil (Cavasini 2007a, 2007b), and evidence of transmission has also been documented in DARC-negative individuals on the island of Madagascar (Menard 2010) and in parts of West Africa (Mendes 2011), suggesting that a subset of *P. vivax* isolates might deploy an alternative pathway for invasion that might have implications for vaccines based on this specific interaction.

ABO blood groups and malaria

ABO, the most important blood group system of humans from a clinical perspective, was first recognized at the beginning of the 20th century (Landsteiner 1901). The ABO blood group system involves the expression of one or two of three different saccharide antigens – A, B, and H –attached to the surface of red blood cells and other body tissues via a variety of glycoproteins and glycolipids (Daniels 2005). The H antigen, expressed in blood group O individuals, is derived by the action of a fucosyl transferase enzyme encoded on chromosome 19; the co-dominant A and B antigens are derived by modification of the H antigen through the action of glycotransferase enzymes that are encoded at the ABO locus on chromosome 9 (Daniels 2005).

Although a link between the ABO blood groups and susceptibility to malaria has been suspected for many years, compelling evidence has been slow to accumulate (Cserti 2007). Soon after the ABO blood group was first recognized, it was recognized that the distribution of these blood groups and their various subtypes varied among populations (Hirschfeld 1919). Although evidence supports the notion that *A* is the ancestral allele (Calafell 2008), blood group O is the predominant phenotype in much of sub-Saharan African and in other malaria-endemic areas (Mourant 1976; Cserti 2007), an observation that is compatible with the hypothesis that O was selected in those areas through a survival advantage against malaria (Mourant 1978; Cserti 2007) and further supported by genetic data suggesting that blood group O results from multiple mutations that arose and were amplified to their current frequencies during a time scale compatible with malaria selection (Cserti 2007; Calafell 2008; Cserti-Gazdewich 2011).

Evidence suggests that the *B* and *O* alleles result from mutations in the *A* gene that occurred at various points between 3.5 million and 0.3 million years ago (Calafell 2008). The *B* allele derives from the most ancient mutation, which results in the transcription of a glycosyl transferase enzyme that results in the addition of D-galactose rather than the normal N-acetyl D-galactosamine to the free terminal of the H-antigen (Yamamoto 1990). The *O* allele arose from mutations that occurred on at least three occasions between 316,000 and 2.5 million years ago (Calafell 2008) that result in the inactivation of the *A* allele, with the result that the H antigen remains unmodified in such individuals (Yamamoto 1990). Four possible blood group phenotypes can therefore result from the inheritance of the *A*, *B*, or O alleles: A, B, AB or O.

Although the relationship between the ABO blood groups and various forms of clinical and asymptomatic malaria has been investigated through multiple studies, until recently the epidemiological data have been inconclusive, as has been summarized by Cserti (2007). However, a number

of studies have been reported that now provide conclusive evidence for a protective effect of blood group O against severe and complicated malaria. In a large case-control study conducted in Mali, Rowe and colleagues found that compared to subjects of the non-O types, blood group O was associated with a protective advantage of 66% (Rowe 2007). In conjunction with a series of other case-control studies conducted in multiple countries, using either serological (Pathirana 2005; Cserti-Gazdewich 2012; Degarege 2012; Rout 2012) or genetically-based typing methods (Fry 2008b; Jallow 2009; Timmann 2012), we now have strong evidence that blood group O protects against severe and complicated malaria but that the degree of this protection can vary between populations, perhaps reflecting differences in the prevalence of other malaria-protective genes, varying transmission levels, or regionally specific parasite-specific factors (Rowe 2009b).

Mechanisms of protection by blood group O

As described by other authors in the section on parasite adhesion in chapter 16, rosetting – an *in vitro* phenotype characterized by the adhesion of uninfected- to malaria-parasite-infected red blood cells to form clumps – has been associated with the pathogenesis of severe and complicated malaria through a number of studies (Rowe 2009a). The ABO blood groups system has been shown to play an important role as a mediator of rosetting. Rosetting is lower, in terms of both prevalence and degree, among subjects with blood group O than non-O, both *ex vivo* and in *in vitro* experiments using either laboratory parasite strains or field isolates (Carlson 1992; Udomsangpetch 1993; Chotivanich 1998; Barragan 2000; Rowe 2007; Rout 2012).

The structural basis for the molecular interactions between *P. falciparum* parasites and ABO blood groups expressed on the red blood cell surface has been described through a series of elegant studies conducted by Vigan-Womas and colleagues (Vigan-Womas 2012). They used a series of techniques, including recombinant *P. falciparum* proteins, to show that binding to the ABO antigens is mediated by a specific subgroup of PfEMP1 of the VarO lineage and that the strength of binding follows the order A followed by B and is weakest in O (Vigan-Womas 2012). The now-strong evidence for a protective effect of blood group O in association with mechanistic data supporting a role for rosetting provides justification for the continued efforts to design therapeutic interventions aimed at preventing or reversing rosette formation as an approach to improving outcome in subjects exposed to natural infection.

However, a study has suggested an alternative or complementary mechanism for the protection afforded by blood group O. In a series of studies conducted using laboratory parasites in red blood cells of different ABO groups, Wolofsky and colleagues found that macrophage-mediated phagocytosis of *P. falciparum*–infected group O red blood cells was significantly more efficient than that of infected group A or B red cells, suggesting that the protection afforded by blood group O may be mediated by more than one mechanism (Wolofsky 2012). Further studies of these mechanisms may improve our knowledge of parasite biology.

Complement receptor-1

In common with other blood group antigens, complement receptor-1 (CR1) is a glycoprotein that is expressed on a range of tissues including red and white blood cells and glomerular podocytes (Fearon 1980; Fearon 1983; Reynes 1985). CR1 plays important roles in the control of complement activation, in the immune clearance of C3b- and C4b-coated immune complexes from circulation, and in phagocytosis (Khera 2009). CR1is subject to two forms of variation: antigenic variation in the form of the Knops (KN) blood group system (Moulds 1991) and variation in the number of copies of the molecule that are expressed on the surface of cells. The KN system is composed of nine antigens that include the McCoy (Mc), Swain Langley (Sl) and York (Yk) blood group systems (Moulds 1991; Rao 1991).

The molecular genetics of the KN system has been fully elucidated (Moulds 2001; Tamasauskas 2001), but the factors determining copy number variation are less clear. Although the trait is under genetic control in European, Asian, and Melanesian populations, in which SNPs in intron 27 and exon 22 of the CR1 gene give rise to low *(L)* and high *(H)* expression alleles (Wilson 1986; Xiang 1999; Cockburn 2004), the same mechanisms do not appear to be operating in populations from sub-Saharan Africa (Herrera 1998; Rowe 2002). Furthermore, CR1 copy number has been shown to vary with age (Waitumbi 2004) and is reduced by a number of factors including intercurrent infections with malaria parasites (Waitumbi 2000; Stoute 2003), making epidemiological studies of this phenotype both more difficult to conduct and more difficult to interpret (Stoute 2005).

CR1 and malaria

CR1 has been implicated in the pathophysiology of malaria infections on the basis of evidence from a number of studies. First, Rowe and colleagues demonstrated that, like the A and B blood group antigens, CR1 can act as a red blood cell receptor for rosetting: red blood cells of the CR1^{null}Helgeson phenotype show a substantially reduced ability to form rosettes (Rowe 1997), soluble CR1 protein inhibits rosetting in CR1 positive red blood cells (Rowe 1997), and rosetting is reduced in the presence of a mAb with specificity for the C3b-binding site of CR1 in both laboratory strains and in field isolates (Rowe 2000). The usual red blood cell receptors for *P. falciparum* parasite invasion are glycophorin molecules (see later in this chapter), but two groups have shown that the CR1 molecule can act as an alternative receptor and that invasion via this pathway can be blocked both by soluble CR1 and by specific monoclonal antibodies (Spadafora 2010; Tham 2010; Awandare 2011), suggesting a further potential target for vaccine development.

CR1 copy number and epidemiological studies of malaria

Studies investigating the association between CR1 copy number and the risk of various forms of clinical malaria have produced inconsistent results. Cockburn and colleagues found a significant protective association of the *L* allele against severe and complicated malaria in Papua New Guinea (Cockburn 2004), but homozygosity for the *L* allele was found to be a risk factor for severe malaria in a study conducted in Thailand (Nagayasu 2001). One possibility is that the discrepancy between the results of these two studies relates to transmission intensity.

In a study conducted in India, Sinha and colleagues studied CR1 copy number variation and malaria susceptibility in populations from a malaria-endemic region in Eastern India and a nonendemic region in Northern India (Sinha 2009). CR1 copy number was significantly lower in the malaria-endemic than in the nonendemic population, an observation consistent with selection for the low-expression phenotype. However, the authors found a contradictory relationship between CR1 and malaria in the two populations: in the nonendemic area, low CR1 expression levels were found to be a risk factor for severe malaria, whereas in the malaria-endemic area high expression was found to be a risk factor for more-severe forms of disease (Sinha 2009).

As discussed by Stoute (2005), it seems possible that the relationship between CR1 copy number and malaria may be characterized by a complex balance among a number of factors including age, transmission intensity, and the different pathophysiological basis for the various forms of severe disease. Further studies are needed before the full picture regarding the role of CR1 copy number in malaria pathogenesis is fully understood.

Knops antigens and clinical malaria

Genetic analysis of the Swain Langly *(Sl)* gene provides strong evidence for natural selection (Barreiro 2008) and, like many malaria candidate loci, the global distribution of various KN blood group antigens follows a pattern consistent with the conclusion that malaria may be the responsible agent (Moulds 2000; Zimmerman 2003; Thathy 2005). Of particular note are the high frequencies

of the Sl2 and McC^b antigens in high-transmission in comparison to non-endemic regions (summarized in (Rowe 2009b)). Nevertheless, to date the clinical relationship between KN blood groups and malaria has only been investigated through two epidemiological studies; no significant effect was found in one (Zimmerman 2003), and a protective effect of Sl2 against cerebral malaria was found in the second (Thathy 2005). Although it is possible that this protective effect relates to rosetting (Rowe 1997), nothing is known about the effect of the various KN blood groups on rosetting under conditions of natural exposure. Further studies investigating the influence of KN blood group variants on various aspects of clinical malaria, preferably in diverse populations, are needed before conclusive answers are forthcoming.

Other blood group antigens and red blood cell membrane proteins

In addition to DARC, ABO, and CR1, malaria has been implicated in the evolution of a wide variety of other blood group antigens and red blood cell membrane proteins.

Glycophorins

The glycophorin (GYP) molecules A to E are a group of red blood cell surface–expressed sialoglycoproteins that are encoded by the *GYP* genes on chromosome 4 (Rearden 1993). As a group, the glycophorins are important receptors for the invasion of red blood cells by *P. falciparum* parasites. Although a role for GYPB and GYPC was demonstrated soon after parasite culture became possible *in vitro* (Pasvol 1982a; Pasvol 1982b; Pasvol 1984), subsequent studies suggest that the more-abundant GYP molecule, GYPA, is the most important of the group in terms of its role as a receptor for *P. falciparum* invasion in nature (Sim 1994; Baum 2003).

Studies of the interaction between malaria parasites and these receptors have led to the identification of a number of molecules expressed at the merozoite stage of development that offer potential targets for vaccine development. For example, the invasion of *P. falciparum* parasites via GYPA involves a specific interaction between the Duffy-binding–like (DBL) domains of the merozoite surface protein EBA-175 and domains within the GYPA molecule (Tolia 2005), whereas invasion using other GYP molecules involves a range of other specific merozoite proteins that include a number of DBL homologues (Mayer 2001, 2002, 2004, 2006, 2009).

GYP mutations and protection from clinical malaria

Sequence analysis suggests that at least some of the *GYP* genes have come under strong evolutionary selection in keeping with pressure from a survival advantage against malaria (Blumenfeld 1995; Baum 2002; Wang 2003), and a number of mutations of the *GYP* genes have risen in frequency in a range of different malaria-exposed populations. For example, a *GYPA* mutation that results in the expression of the hybrid GYPA-GYPB Dantu protein occurs at low frequencies in parts of southern Africa (Tanner 1980), the allele frequency for a null mutation of *GLPB* exceeds 50% in one population of Congolese Pygmies (Fraser 1966), and an exon 3 deletion within the *GYPC* gene that causes the Gerbich-negative blood group phenotype (Colin 1989; High 1989) is common in malaria-endemic but rare in nonendemic parts of Papua New Guinea (Booth 1972a; Booth 1972b; Booth 1972c).

Nevertheless, few data are available regarding the clinical relevance of such polymorphisms. Although the Dantu antigen is associated with reduced invasion of *P. falciparum* parasites *in vitro* (Field 1994) no clinical studies have reported the effect of this polymorphism under conditions of natural exposure. Similarly, despite experiments showing that *P. falciparum* parasites cannot invade Gerbich-negative red cells via the *GYPC* pathway (Maier 2003) studies conducted to date have not revealed a protective effect against clinical malaria (Patel 2001; Patel 2004; Fowkes 2008b). The likely effectiveness, therefore, of vaccines and therapies aimed at blocking invasion via specific GYP pathways remains a matter for speculation.

Ovalocytosis

Ovalocytosis is a condition characterized by morphologically abnormal red blood cells. Although the condition can be caused by a number of genetic lesions, the most common form, Southeast Asian ovalocytosis (SAO), occurs in subjects who are heterozygous for the 27-bp *SLC4A1Δ27* deletion in the gene encoding the RBC membrane protein band 3. Despite reaching very high frequencies in some populations, most notably in malaria-endemic parts of Papua New Guinea, the homozygous state is thought to be lethal (Liu 1994; Genton 1995; Allen 1999). This suggests that, like the β^s globin mutation that causes sickle cell disease (see below), *SLC4A1Δ27* is an example of a balanced polymorphism – a condition that has risen to high frequencies in specific populations through positive selection for heterozygotes at the expense of a selective disadvantage in homozygotes. The distribution of *SLC4A1Δ27* mirrors that of malaria within Papua New Guinea, reaching 35% in the areas of highest transmission (Mgone 1996).

Although no evidence has been found that of *SLC4A1Δ27* protects against asymptomatic (Cattani 1987; Kimura 2002; Shimizu 2005) or uncomplicated *P. falciparum* or *P. vivax* malaria (Patel 2001; Lin 2010), a specific protective effect of *SLC4A1Δ27* against cerebral malaria was seen in two case-control studies (Genton 1995; Allen 1999). In subsequent studies conducted to investigate potential mechanisms for this specific protective effect, Cortes and colleagues showed that whereas *SLC4A1Δ27* red blood cells were resistant *in vitro* to invasion by many *P. falciparum* isolates, they were not resistant to all, suggesting that protection might result from resistance to a subset of parasites that are particularly associated with severe disease (Cortes 2004). Furthermore, the investigators found evidence for increased cytoadherence of *P. falciparum*–infected *SLC4A1Δ27* red cells to the endothelial receptor CD36 in a flow-based assay.

CD36 is not expressed on neurovascular endothelium, and cytoadherence to CD36 has not therefore been implicated in the pathogenesis of cerebral malaria (Turner 1994). One interpretation of this finding, therefore, is that increased cytoadherence to non-cerebral sites might distract *P. falciparum*–infected *SLC4A1Δ27* red cells away from cerebrovascular sites and reduce the occurrence of cerebral malaria.

Finally, a report has reopened the case for the malaria-protective effect of *P. falciparum*–infected *SLC4A1Δ27*. In an analysis of data from two cohort studies and a case-control study, Rosanas-Urgell and colleagues found unexpected evidence for a marked protective effect of *SLC4A1Δ27* against clinical *P. vivax* infections (Rosanas-Urgell 2012). This observation leads to the alternative possibility that the *SLC4A1Δ27* mutation may have come under selection for a survival advantage against *P. vivax* malaria. Although most clinical descriptions of *P. vivax* paint a picture of a disease that, while unpleasant, is associated with relatively low mortality, a number of studies suggest that *P. vivax* can result in a severe and complicated illness not dissimilar to that caused by *P. falciparum* (Genton, 2008; Tjitra 2008). Moreover, as discussed above, the DARC-negative phenotype provides a precedent for genetic selection for a survival advantage against *P. vivax* malaria. Further studies investigating the mechanism by which the *SLC4A1Δ27* might result in protection against both *P. falciparum* and *P. vivax* disease are awaited with interest.

Hemoglobinopathies

Some of the earliest and best-described malaria-protective polymorphisms are the hemoglobinopathies. These conditions fall into two broad groups: disorders characterized by the abnormal structure of hemoglobin, including hemoglobin S (HbS), hemoglobin C (HbC), and hemoglobin E, and the thalassemias, conditions characterized by the reduced production of normal forms of the α- or β-globin subunits of hemoglobin.

Structural hemoglobin variants

Normal adult hemoglobin is a complex molecule made up of a number of components that include two α- and two β-globin polypeptides, which surround a central iron-containing heme moiety (Perutz 1963). The three structural disorders of hemoglobin about which most are known in the context of malaria are HbS, HbC, and HbE. All three conditions are caused by mutations in the *HBB* gene on chromosome 11, which codes for β-globin. A larger number of studies have been reported that relate to HbS than to either HbC or HbE.

Sickle hemoglobin (HbS)

A number of different investigators first suspected a protective relationship between HbS and malaria more than 60 years ago. Beet (1946, 1947), Brain (1952), and Allison (Allison 1954) all noticed a correlation between the prevalence of "sickling" in a number of African populations and the endemicity of *P. falciparum* malaria. Subsequent studies have shown that this is true at a global scale (Livingstone 1973, 1985) and that, at least within Africa, this relationship is highly significant on the basis of a Bayesian geostatistical model (Piel 2010).

HbS is caused by a non-synonymous SNP in the 17th nucleotide of the *HBB* gene that results in the substitution of valine for the usual glutamic acid in position 6 of the β-globin chain (β^s) (Pauling 1949). Haplotype analysis suggests that this SNP has arisen, and has been independently amplified to its current population frequencies, on at least two, and possibly on more, occasions (Kulozik 1986; Flint 1998). The high allele frequencies for β^s that are seen in wide swathes of the malaria-endemic world, which exceed 15% in areas of highest transmission (Piel 2012), are even more remarkable given the probability that, historically, the homozygous state for the β^s variant (HbSS; sickle cell anemia) was universally fatal during the course of early childhood (Grosse 2011). This means that HbS is now the best-known example of a balanced polymorphism in humans.

Strong evidence that malaria is the agent responsible for selection of β^s comes from numerous clinical studies of various design that have been conducted in several countries over many years (for examples see Willcox 1983b; Hill 1991; Williams 2005a, 2005b; Jallow 2009). In a meta-analysis of published studies, Taylor and colleagues found that overall, heterozygosity for the β^s mutation (HbAS; sickle cell trait) is associated with greater than 30% protection against uncomplicated episodes of clinical malaria and greater than 90% protection against severe and complicated forms of the disease (Taylor 2012). Moreover, the strong and specific protection afforded by HbAS against all forms of clinical malaria have proved useful in quantifying the contribution of malaria to other childhood diseases such as malnutrition (Nyakeriga 2004; Kreuels 2009) and invasive bacterial infections (Scott 2011). Of further interest, studies suggest that although HbAS subjects are personally protected against *P. falciparum* disease, they are more infectious to the *Anopheles* vectors, and this personal advantage is balanced not only by the cost of homozygosity but also by the cost to the wider community in terms of increased malaria transmission (Gouagna 2010; Lawaly 2010).

Finally, the role of malaria in the high mortality that is associated with HbSS remains unclear. Given the strong protection afforded by HbAS it has been speculated that HbSS should provide an even greater degree of protection (Makani 2010; McAuley 2010). A review of the evidence suggests that this may indeed be the case but that when HbSS subjects do become infected with malaria, the consequences are so severe that any protective advantage is more than offset by the increased mortality that is associated with such infections (Williams 2011b).

Hemoglobin C

Like HbS, HbC is a structurally abnormal form of hemoglobin caused by mutations in the *HBB* gene. HbC is clinically benign in both its heterozygous (HbAC) and homozygous (HbCC) forms but results in a variant form of sickle cell disease when co-inherited with HbS (HbSC) (Steinberg 2009).

Although like HbS, the allele frequency of HbC correlates with malaria transmission, the condition is restricted historically to West and Northwest Africa and to migration routes therefrom (Modiano 2008). Population genetic data suggest that the β^C allele arose before the β^s allele and has taken considerably longer to reach its current incidence (Modiano 2008). Fewer epidemiological studies have investigated the protective effect of HbC, but the balance of evidence suggests that HbCC is associated with a considerably greater degree of protection (~70%) from severe forms of *P. falciparum* malaria than is HbAC (~17%) and that neither form is consistently protective against less-severe forms of infection (Taylor 2012).

Mechanism of protection by HbS and HbC

Despite what is now overwhelming evidence for the protective effect of HbAS against all forms of clinical *P. falciparum* malaria, the precise mechanisms by which it results in this protection remain unknown (Williams 2011a). A number have been suggested, some of which are supported by reasonable scientific evidence, but which of these numerous mechanisms is most important in nature remains a matter of continued speculation. Early studies suggested that when infected, red blood cells from HbAS subjects were prone to sickle and were therefore more likely to be removed from circulation by the spleen (Luzzatto 1970; Roth 1978). Subsequent studies showed that *P. falciparum* parasites were also less able to be grown *in vitro* in red blood cells containing either HbS or HbC, an observation attributed to oxidant damage (Friedman 1978; Friedman 1979a, 1979b, 1979c, 1979d; Pasvol 1978) and were also more vulnerable to removal from circulation by monocytes (Ayi 2004).

A number of investigators have suggested that rather than being entirely innate, the protective effect of HbS might also involve an immunological component. For example, both Williams and colleagues and Gong and colleagues have shown that the degree of protection afforded by HbAS increases with age among cohorts of children followed for malaria infections in Kenya (Williams 2005a) and Uganda (Gong 2012), respectively. Although this may relate to accelerated immune responses to proteins expressed on the red blood cell surface (Marsh 1989; Cabrera 2005), the evidence supporting this hypothesis is mixed (Tan 2011), and further studies are required to investigate this hypothesis more definitively.

Studies have suggested an alternative or additional explanation for the protective advantage of both HbS and HbC. Display of the *P. falciparum* molecule PfEMP1 on the surface of red blood cells containing either form of variant hemoglobin has been reduced in studies conducted *in vitro* (Fairhurst 2005; Cholera 2008). Similarly, such red blood cells have a reduced ability to cytoadhere to a range of endothelial receptor molecules in a model system, suggesting that protection may result from a decreased ability of such red blood cells to sequester in deep vascular tissues (Fairhurst 2005; Cholera 2008). This hypothesis has been exploited to investigate the biology of PfEMP1 transport within the red blood cell. Using cryoelectron tomography, Cyrklaff and colleagues found that *P. falciparum* parasites generate a host-derived actin cytoskeleton within the cytoplasm that connects the Maurer's clefts with the host cell membrane and to which transport vesicles attach. This process was inhibited in both HbCC and HbAC red blood cells, a phenomenon that could be reproduced in HbAA red cells by the addition of products of hemoglobin oxidation that are enriched in HbC-containing cells (Cyrklaff 2011). Such information may point to new avenues in the development of malaria therapies that result from the study of host resistance factors.

Hemoglobin E

Hemoglobin E (HbE) is a third structural β-globin variant that has risen to high population frequencies in a number of malaria-endemic countries. HbE is particularly common in South and Southeast Asia, where genetic studies suggest that the mutation may have arisen very recently, and certainly within the last 5000 years (Ohashi 2004). In common with many of the genes described above, this observation is in keeping with a selective advantage for HbE in the face of malaria transmission.

Like HbC, HbE is generally a benign condition unless inherited in conjunction with other disorders such as β-thalassemia (Steinberg 2009). There is some evidence from *in vitro* studies that HbE red blood cells are partially resistant to *P. falciparum* invasion (Chotivanich 2002), but few studies have investigated the protective effect of HbE *in vivo* (Taylor 2012). Although one study, conducted in adults, suggested a reduced severity of disease among HbAE patients admitted with *P. falciparum* malaria (Hutagalung 1999), a second found evidence for an increased risk of *P. vivax* malaria among compound HbE/β-thalassemia heterozygotes. Although it seems likely that, in common with other structural β-globin variants, HbE has risen to its current prevalence through malaria selection, more work is needed before we will have a clear understanding of the magnitude of protection against which forms of malaria or of the mechanisms that might be involved.

The thalassemias

Considerably more is known about the relationship between the thalassemias and malaria risk than about many genetic conditions. The thalassemias are characterized by the reduced production of the globin molecules that normally combine to form hemoglobin (Weatherall 2002; Steinberg 2009). They fall into two broad categories, the α- and β-thalassemias, depending on which of the globin molecules are affected. As a group, the thalassemias have arisen from genetic events that have occurred on numerous occasions, and they have been amplified to their current population frequencies through natural selection during a time course compatible with selection by malaria (Flint 1998).

The thalassemias were the subject of the original "malaria hypothesis" put forward by Haldane more than 60 years ago – the suggestion that the current frequencies of what we now know as β-thalassemia might result from its selection through a survival advantage from malaria (Haldane 1949). Like many of the other malaria-protective genes described above, the distribution of the thalassemias is in close agreement with that of historic malaria transmission at both regional (Siniscalco 1961; Flint 1986; Modiano 1991; Enevold 2007) and global scales (Livingstone 1985; Cavalli-Sforza 1994; Flint 1998). Moreover, the effect of selection has been so strong that throughout a wide swathe of the malaria-endemic world, people who carry one or the other form of thalassemia outnumber those who do not (Flint 1998), a fact that makes these the most common genetic polymorphisms that have so far been identified in humans (Weatherall 1981).

Despite strong population genetic evidence for selection of β-thalassemia by malaria, few clinical studies have been reported from which to judge the magnitude or specificity of this effect (Taylor 2012). The studies conducted by Willcox and colleagues in Liberia in the 1980s remain some of the only studies that have addressed this question epidemiologically. They found some evidence for protection by β-thalassemia at the level of *P. falciparum* densities through cross-sectional surveys (Willcox 1981; Willcox 1983a) and evidence for a protective effect against admission to hospital with malaria of approximately 50% on the basis of a single case-control study (Willcox 1983b).

Conversely, protection by α-thalassemia has been investigated through numerous studies of various designs in multiple populations (Taylor 2012). Studies investigating the incidence of uncomplicated malaria have yielded varied results that have included protective associations in some (Enevold 2005; Wambua 2006b; Veenemans 2008), no association in others (Crompton 2008; Lin 2010; Ellis 2011), and an association with increased risk in several (Williams 1996; Veenemans 2011). Nevertheless, studies of more-severe malaria have generally pointed to a significant degree of protection that is more marked in homozygotes than in heterozygotes (Allen 1997; Williams 2005d; Wambua 2006a; May 2007; Manjurano 2012). In a metaanalysis of the available data, Taylor and colleagues found no evidence for a significant effect of α-thalassemia on uncomplicated malaria but an average of 17% protection by heterozygous and 37% protection by homozygous forms of the condition (Taylor 2012). On balance, such studies have suggested that this protection is greatest against forms of severe malaria that are associated with anemia, an observation that may be of relevance to the mechanisms involved.

Mechanisms of protection afforded by the thalassemias

Early studies suggested that the thalassemias might result in malaria protection through an immunological mechanism. Luzzi and colleagues found that *in vitro*, antibody binding to the surface of *P. falciparum*–infected red blood cells incubated in serum from malaria-immune subjects was greater in thalassemic than in normal subjects (Luzzi 1991). Later studies confirmed this finding and suggested that this was not related to increased expression of parasite-derived proteins but might relate to increased recognition of altered red blood cell structural proteins such as band 3 (Williams 2002). Another found that, like HbS and HbC (Fairhurst 2005; Cholera 2008), the display of PfEMP1 is altered on the surface of *P. falciparum*-infected thalassemic red blood cells, an observation that might translate to lower levels of cytoadherence *in vivo* (Krause 2012). Finally, in a study conducted in Papua New Guinea, Cockburn found that α-thalassemia was associated with reduced red blood cell surface expression of CR1, suggesting that the condition might result in malaria protection via a mechanism related to rosetting (Cockburn 2004).

A number of theories have been advanced that could explain why α-thalassemia might result in specific protection from severe malaria anemia. Fowkes and colleagues have suggested that the higher red blood cell counts that are associated with α-thalassemia may buffer against the decline in hemoglobin that is associated with the evolution of severe malaria anemia (Fowkes 2008a), and Veenemans and colleagues suggested that an immunological mechanism may be involved (Veenemans 2008). In two cohorts of children followed for malaria events in Kenya and Tanzania, they found that the greatest falls in hemoglobin occurred in children with raised C-reactive protein (CRP) levels, a marker of inflammation, and that α-thalassemia was protective against this inflammatory form of the disease. A large number of hypotheses have therefore been advanced that might potentially explain various aspects of the protective effect of the thalassemias. Which, if any, of these is most important in nature remains an open question.

G6PD deficiency

In addition to the structural red blood cell protein defects described above, there is also evidence for selection by malaria for polymorphisms affecting the production of enzymes that protect these cells against oxidant stress. Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked housekeeping enzyme involved in the pentose–phosphate pathway and is important in a number of metabolic processes (Cappellini 2008). More than 100 different genetic lesions have been described that can result in protein variants that display different levels of G6PD enzyme deficiency (Cappellini 2008). The most-severe forms, which are associated with very low levels of residual G6PD activity, can result in episodes of intravascular hemolysis on exposure to oxidant substances such as fava beans (Waller 1957; Sansone 1958) or drugs that include the antimalarial agents primaquine (Beutler 1959) and dapsone (Pamba 2012). The commonest enzymopathy of humans, allele frequencies for G6PD deficiency exceed 30% in many parts of sub-Saharan Africa (Howes 2012).

A selective advantage of G6PD deficiency against malaria is supported by its multicentric origins and the close correlation between the numerous causal polymorphisms and malaria both at regional (Allison 1960; Siniscalco 1961, 1966; Ganczakowski 1995; Phompradit 2011) and global (Livingstone 1973; Singh 1973; Mourant 1976; Nkhoma 2009; Howes 2012) scales. Genetic analysis of some of these variants suggest that they arose and have been amplified to their current frequencies through positive selection over a time scale compatible with selection by malaria. For example, the antiquity of the African *A-* variant has been estimated at between 1000 (Slatkin 2008) and 6360 years (Tishkoff 2001), whereas that of the *Med* and *Mahidol* variants have been estimated at 3330

(Tishkoff 2001) and 1580 (Louicharoen 2009) years, respectively. Nevertheless, available epidemiological data regarding G6PD deficiency are somewhat confusing.

Good evidence has accumulated to suggest that G6PD deficiency confers some protection against *P. falciparum*; however, the degree to which protection is afforded to males and females has varied between different studies. In an early study, conducted in Tanzania, Allison and Clyde found evidence for protection, in the form of reduced parasite densities, in both males and females with G6PD deficiency (Allison 1961). However, in later studies, Bienzle and colleagues found that protection was limited to heterozygous females in Nigeria (Bienzle 1972), and subsequent case-control and cohort studies have reported no protection by G6PD deficiency against severe malaria in Nigeria (Martin 1979), similar degrees of protection in hemizygous boys and heterozygous girls in The Gambia and Kenya (Ruwende 1995; Clark 2009), and restriction of protection to hemizygous males (Guindo 2007) or deficient or heterozygous females in a variety of other settings (Johnson 2009; Manjurano 2012). Finally, the results of a study conducted in Afghanistan suggest that G6PD deficiency may also be protective against *P. vivax* malaria (Leslie 2010).

The seemingly contradictory findings from different populations may reflect a variety of factors, including differences in study design; allelic heterogeneity at the G6PD locus, a factor that could potentially lead to misclassification of G6PD deficiency in studies that rely on genotype results (Clark 2009; Johnson 2009); and interactions with other alleles, including HbS (Guindo, 2011). On the basis of theoretical considerations, supported by data from a subset of studies conducted to date (Bienzle 1972; Clark 2009), Luzzatto argues that that the most likely model for the protective effect of G6PD is selection for female heterozygosity (Luzzatto 2012). Hopefully, this debate will be clarified in the very near future by data from definitive multicenter studies (MalariaGEN).

In the absence of definitive data regarding which aspect of G6PD deficiency is associated with protection against which types of clinical malaria, it is difficult to determine the precise mechanisms involved. *In vitro* studies have shown slower rates of *P. falciparum* growth in G6PD deficient red blood cells (Roth 1983; Miller 1984; Ginsburg 1996), an observation that may relate to the accumulation of oxidative products that are normally metabolized in G6PD sufficient cells. Of particular note, Luzzatto and colleagues found that within red blood cells from G6PD deficient heterozygous females, parasite growth was between 2 and 80 times greater within G6PD normal than in G6PD deficient red cells (Luzzatto 1969). In one study investigating the acquisition of malaria-specific immune responses, Courtin and colleagues found that G6PD deficient subjects developed antibodies to merozoite surface proteins more slowly than G6PD normal subjects, an observation that might result from more-rapid removal of parasites by innate mechanisms that reduce exposure of the immune system to parasite antigens (Courtin 2011). Observations by Cappadoro and colleagues may be relevant in this regard; they found that *P. falciparum*–infected G6PD deficient red blood cells are more rapidly cleared from circulation by monocytes (Cappadoro 1998) in a fashion analogous to that of red blood cells from subjects with HbAS and β-thalassemia (Ayi 2004), suggesting a potentially common mechanism for malaria protection across a range of different conditions.

Non–red blood cell polymorphisms

Although the majority of the malaria-protective polymorphisms that have been described to date relate to the red blood cell, there is also evidence for malaria selection of polymorphisms in genes involved in a wide range of other pathways. Examples include genes involved in immune regulation, cytokine production, protection from oxidant stress, and production of proteins expressed on the endothelium and other tissues. A summary of some of these genes and the evidence supporting them is provided in Table 17.2. Such a table can never be exhaustive: associations have been

Table 17.2 Examples of candidate malaria-association genes relating to non–red blood cell products.

Group	Gene	Function	Evidence	References
Immune pathways	*HLA-B/DR*	Antigen presentation	HLAB53 allele frequency high in malaria-endemic parts of sub-Saharan Africa, HLAB53 protective in a case-control study, HLADRB1 variants protective in a case-control study	[160, 252]
	FCGR2A/B	Clearance of antigen–antibody complexes	Case-control evidence,associated with reduced parasite densities (Kenya), increased cerebral malaria (Thailand), correlations with malaria, functional data	[253–257]
	IFNG	Proinflammatory cytokine	Plasma levels associated with disease, SNPs associated with risk	[258–260]
	IFNGR1	Cytokine receptor	Clinical protection against cerebral malaria	[261]
	IL1A/B	Proinflammatory cytokine	Suggestive clinical data from the Gambia	[262]
	IL10	Antiinflammatory cytokine	Clinical protection in some studies	[263, 264]
	IL4	Cytokine promoting B-cells	Anti-malarial antibody levels and parasite prevalence associate with IL4 polymorphisms	[265, 266]
	MBL2	Complement activation	Clinical studies variable	[267]
	NOS2A	Generates NO	Evidence that nitric oxide involved in pathogenesis of severe malaria, clinical studies of genetic variants conflicting	[268–271]
	TNF	Proinflammatory cytokine	Clinical protection in some studies, data conflicting	[272–274]
	TLR1/4/6/9	Pathogen recognition	Co-distribution with malaria and signatures of genetic selection, clinical evidence still lacking	[275–278]
	NOD1/2	Pathogen recognition	Murine models suggest involvement in pathogenesis, clinical data few	[279]
	CD40LG	B-cell stimulation	Case control evidence for protection and genetic analysis showing recent selection	[280, 281]
Cytoadherence	*CD36*	Endothelial receptor	Protection from severe malaria by a null mutation in one study, susceptibility in a second	[282, 283]
	ICAM1	Endothelial receptor	Protection from severe malaria by ICAM-1Kilifi in some studies but not in others.	[284, 285]
	PECAM1	Endothelial receptor	Clinical associations in some studies but not others	[286, 287]
Oxidant stress	*HP*	Binds free hemoglobin in plasma	Conflicting data regarding haptoglobin polymorphisms and malaria from multiple studies	[288–290]
	HO-1	Catabolism of free heme	Upregulated in severe disease, associated with severe disease in children, mouse models	[25, 291, 292]

proposed with many hundreds of genes and gene pathways, and the list is constantly growing. However, in the vast majority of cases the evidence supporting an association with malaria susceptibility is less conclusive for these genes than it is for the genetic variants of red blood cells. It must be said that in many cases the evidence is derived from a limited number of clinical studies, many bedeviled by small sample sizes, and all too often producing conflicting results. Greater clarity will hopefully be achieved with regard to many of these candidates when the results of a major multicenter study are reported that involves candidate-gene case-control studies conducted in multiple countries throughout the malaria-endemic world (MalariaGEN).

Concluding remarks

The relationship between malaria and host genetics has come a long way since Haldane first suggested that β-thalassemia may have risen to its current population frequencies as a result of natural selection by *P. falciparum* disease (Haldane 1949). Like so many areas of science, the story has not been as straightforward as might once have been anticipated, unexpected interactions between genes (Williams 2005c; Penman 2009, 2011; Guindo 2011) and allelic heterogeneity being just two potential explanations. Nevertheless, the case regarding a number of genes, many affecting various aspects of the red blood cell, is now cast iron. In the case of the DARC blood group antigens, the observation that DARC-negative subjects are refractory to *P. vivax* infections has spawned a wide range of important scientific discoveries that have culminated in the development of a new vaccine, giving hope that observations regarding other malaria-protective genes will similarly result, eventually, in novel approaches to prevention and treatment.

Rapid developments in genomic and proteomic science mean that the opportunities for bringing this dream to reality have never been better. For the first time, we now have the ability to screen the genome for thousands of genes simultaneously with a view to identifying novel associations (Jallow 2009; Timmann 2012) and to study the expression profiles of genes from both host and parasite in a single experiment (Idaghdour 2012). Such developments are already bearing fruit, with the identification of a number of promising new proteins that warrant further investigation. One such protein is the Ok blood group antigen basigin, a protein that has been implicated as a receptor for the *P. falciparum* parasite ligand PfRh5, a protein that is essential for blood-stage growth (Crosnier 2011; Wanaguru 2013). The opportunities to translate the lessons learned from the study of host-protective factors into clinically useful solutions have never been greater.

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CHAPTER 18

The immune response in mild and severe malaria: Two sides of the same coin

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Malaria is an infectious disease that afflicts nearly 500 million people each year in parts of the world that are least equipped to cope with its devastation, including sub-Saharan Africa. The vast majority of malaria cases are uncomplicated and not life threatening. However, in a small portion of cases, almost exclusively in young children, the infection is severe, resulting in nearly one million deaths each year in Africa alone. At present we have neither a vaccine to prevent malaria nor adjunctive therapies to treat children with severe disease. Clearly, understanding the immune mechanisms at play in uncomplicated and severe disease would benefit efforts to develop both vaccines and adjunctive therapies. Here we review briefly what is known about the acquisition of immunity to malaria and then focus on the less well understood immune mechanisms at play in severe disease. A theme that we will develop is that in both uncomplicated and severe malaria the ability to control inflammation induced by the parasite is central to the control of disease. We speculate that the ability to control inflammation is dependent on an individual's genetic background and pathogen environment and although essential for survival, control of inflammation may impede the efficient acquisition of protective adaptive immunity.

The picture of the acquisition of resistance to uncomplicated and severe malaria: Framing the questions

Immunity to uncomplicated malaria

Malaria is a disease caused by infection with intracellular Apicomplexa parasitic protozoa that are members of the genus *Plasmodium*. The most deadly species of *Plasmodium* that infects humans is *P. falciparum* that prevails in Africa. *Plasmodium* infections occur in two sequential stages: an asymptomatic liver stage in which parasites infect hepatocytes and a symptomatic blood stage in which parasites infect red blood cells (RBCs). Malaria is typically clinically defined as a fever or other symptoms of malaria in association with asexual parasites in the blood. Nearly all children living in malaria endemic areas are susceptible to disease following parasite infection. Depending on the intensity of malaria transmission, children remain susceptible to malaria until late childhood or early adolescence at which time individuals transition to a malaria resistant state and no longer, or rarely, suffer from clinical disease (Langhorne 2008). It is remarkable that resistance to malaria develops only after years in malaria endemic areas even in areas of high malaria transmission where children can be exposed to hundreds of infectious mosquito bites each year. The length of time required to develop resistance to malaria is particularly striking when compared to the rapid

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acquisition of immunity to many viral diseases after a single infection including measles, rubella and smallpox (Breman and Henderson 2002; Gafafer 1935; Horstmann 1985). Another feature of malaria immunity is its short-lived nature in young children. For example, the half-lives of the IgG antibody responses specific for five parasite antigens measured in children in Africa were on the order of days (Kinyanjui 2007) to months (Akpogheneta 2008; Cavanagh 1998; Fonjungo 1999; Fruh 1991; John 2002; Muller 1989; Ramasamy 1994; Taylor 1996; Weiss 2010). This short half-life of malaria-specific responses is in stark contrast to the calculated half-lives of IgG responses to tetanus vaccine (11 years) and the measles vaccine (>300 years) both given to young children (Amanna 2007; Amanna and Slifka 2010). Even though *P. falciparum* antibody responses are short-lived in children, by adulthood individuals in endemic areas acquire stable levels of *P. falciparum*-specific antibodies that are protective (Deloron and Chougnet 1992). Current evidence indicates that even though resistance to disease is acquired with time and malaria exposure, resistance to infection is rarely if ever acquired such that adults living in an endemic area frequently have asymptomatic infections (Marsh and Kinyanjui 2006; Noubouossie 2012; Tran 2013).

Given this picture of the acquisition of malaria immunity two major unanswered questions are: What constitutes immunity to malaria? And Why does it take so long to develop? At present we have no vaccine for malaria (Crompton 2010; Riley and Stewart 2013) and clearly answers to these questions should benefit efforts to develop an effective vaccine.

Immunity to severe disease

The picture of susceptibility to severe disease and acquisition of resistance is less clear. Severe disease in children is complex and most likely multifactorial encompassing three overlapping syndromes, cerebral malaria, metabolic acidosis/respiratory distress and severe anemia (Marsh 1995; von Seidlein 2012). Nearly all severe disease occurs in African children under the age of five (Marsh 1995; von Seidlein 2012). Although essentially all children in this age group are susceptible to malaria, in the vast majority of these children malaria is uncomplicated and resolves with time. Today, in the era of relatively cheap, available anti-malarial drugs, malaria becomes severe and life threatening in only approximately 1% of young children, with a mortality of 15–20%. However, the number of children estimated to have died from malaria prior to the advent of inexpensive anti-malarial drugs is calculated to be considerably higher, approximately 18% (Molineaux 1985). These facts have two important implications. Firstly they suggest that a much larger proportion of young children are susceptible to severe disease, minimally 18% and secondly, most susceptible children, all but 1%, become resistant to severe disease when anti-malarials are available. The difference in the prevalence of severe disease in the presence or absence of anti-malarials (1% versus 18%) is an interesting phenomenon in itself that deserves further study. However, the phenomenon also makes studying the basis of susceptibility to severe disease challenging because the majority of susceptible children would be classified as resistant in the current day.

It is not clear how resistance to severe disease is acquired or, if the protective immune mechanisms at play in uncomplicated and severe disease are similar. It is possible that children who develop severe disease are relatively naïve to malaria, having little exposure early in life. This begs the question: How many malaria infections are necessary to achieve protection from severe disease? The answer is not clear as determining the risk of severe malaria in relationship to infections requires large cohorts to capture the relatively rare cases of severe disease and such studies have simply not been done. However, a recent nested case control study in Kenya provided evidence that children who suffer severe malaria have had more infections early in life as compared to community controls (Lundblom 2013). Severe disease is less frequent in children living in areas of high versus low malaria transmission leading to the hypothesis that resistance to severe disease may be acquired more rapidly when infections occur in infants under the cover of maternal anti-malarial antibodies

(Gupta 1999). Based on models of the relationships of exposure to infection and resistance to severe disease, resistance to non-cerebral severe malaria has been proposed to occur after only a few infections, even after a single infection in children protected by passively acquired maternal antibodies (Gupta 1999). If so, this would suggest that the acquisition of immunity to uncomplicated versus severe disease are quite different or at very least occur over a very different time scale.

Given this picture of severe disease, important unanswered questions include: What molecular mechanisms underlie immune resistance to severe disease? Why are only a fraction of children who are susceptible to malaria susceptible to severe disease? At present we have no effective adjunctive therapies to treat severe disease (Miller 2013) and clearly answers to these questions would benefit efforts to develop such therapies.

Immunity in uncomplicated malaria

One useful framework in which to discuss the acquisition of malarial immunity is the life cycle of the parasite, *P. falciparum* (Figure 18.1). The infection begins when a parasite infected female *Anopheles* mosquito probes an individual's skin as she prepares to take a blood meal. Her saliva contains a highly motile differentiated form of the *P. falciparum* parasite called a sporozoite and a small number of these (10–50) are injected into the skin. Recent studies have shown that a significant portion of sporozoites remain in the skin for hours if not days (Gueirard 2010). It is not known if the immune system responds to either the mosquito's saliva or to the sporozoites in the skin although there is precedence for both responses in other insect-transmitted parasitic infections (Kamhawi 2000). Sporozoites or sporozoite antigens have been shown to enter the draining lymph nodes from the skin where they are presented by dendritic cells (DCs) to initiate $CD8^+$ T cell responses (Chakravarty 2007). To continue the infection sporozoites in the skin enter the blood and travel to the liver. In the liver the sporozoites invade a small number of hepatocytes. There are no clinical symptoms during the liver stage of the infection and individuals do not know that they are infected at this point. There is no evidence that immunity is acquired in humans to the liver stage of the infection through natural infection (Marsh and Kinyanjui 2006; Tran 2013). Although sterile immunity to infection is not acquired through natural infection, it is possible that immune mechanisms that reduce the number of sporozoites that reach or pass through the liver may have some influence on the time that lapses before the peripheral blood parasitemia reaches levels sufficient to cause symptoms (Greenwood 1991). Short extensions of this period even by a day or two may provide an advantage to blood-stage immune mechanisms in controlling the infection. Indeed, it has been suggested that the partial protection afforded by current liver stage vaccines in clinical trial may be due to enhanced blood stage immunity due to delayed blood stage infection (Bejon 2011).

Although sterile immunity to the liver stage is not naturally acquired, paradoxically, in mice and in humans experimental infections with live, radiation- or genetically-attenuated sporozoites or unattenuated live sporozoites under the cover of anti-malarial treatment have been shown to induce sterile protection against liver stage infections (Butler 2012). The phenomenon was first discovered in a mouse model in which immunization with irradiated *Plasmodium* sporozoites induced sterile protection against live parasite challenge. Protection was shown to be mediated, in part, by $CD8^+$ T cells specific for the circumsporozoite (CS) protein that covers the surface of the sporozoites (Nussenzweig 1967; Romero 1989). CS was recently shown to be presented by DCs in the skin draining lymph nodes to initiate $CD8^+$ T cell responses (Chakravarty 2007; Obeid 2013). The primed CS-specific $CD8^+$ T cells then exit the lymph node and migrate to the liver where they eliminate infected hepatocytes that express processed CS peptides presented on MHC class I molecules (Chakravarty 2007). These observations spurred efforts to develop what is today the leading malaria vaccine candidate, RTS,S that is composed of the hepatitis B virus surface antigen engineered to contain regions of the CS protein (Regules 2011). However, the interim results of an ongoing phase

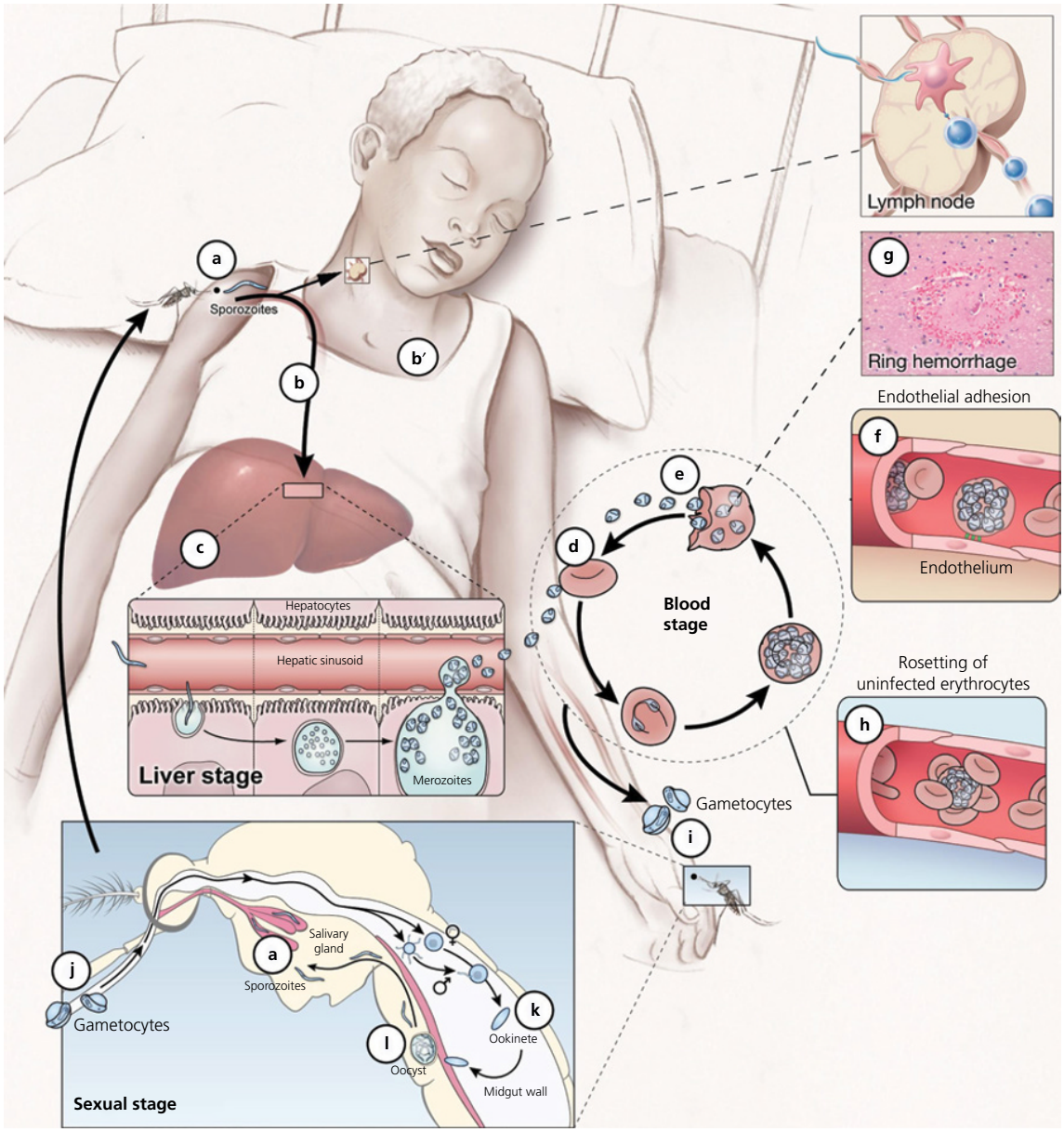

Figure 18.1 The *P. falciparum* life cycle. The *P. falciparum* life cycle in humans includes the asymptomatic liver stage; the blood stage which causes disease; and the sexual gametocyte blood stage which infects mosquitoes that transmit the parasite. Infection begins when *Anopheles* mosquitoes inject sporozoites into the skin where they may persist for days **(a).** Sporozoites migrate from the skin via the blood to the liver and invade a small number of hepatocytes **(b).** Some sporozoites migrate to the draining lymph nodes where sporozoites antigens can be presented by dendritic (DCs) to $CD8^+$ T cells that then migrate to the liver (**b′**). Each sporozoite gives rise to tens of thousands of asexual parasites called merozoites **(c).** Approximately one week after hepatocyte invasion merozoites exit the liver into the bloodstream and begin a 48 h cycle **(d)** of RBC invasion, replication, RBC rupture, and merozoites release **(e).** Symptoms only occur during the blood-stage and can begin as early as 3 days after the release of merozoites from the liver. Once inside RBCs

3 clinical trial in African children showed that RTS,S conferred only short-lived protection against disease in approximately 30% of infants (Agnandji 2012), and did not protect against infection (Alonso 2004; Bejon 2008). Efforts are also continuing to expand on the original observation of sterile immunity induced by attenuated sporozoites to develop whole attenuated sporozoites vaccines (Butler 2012). Analyses of immune parameters that correlate with protection in experimental infections and in vaccine trials should shed light on immune mechanisms at play in the liver stage of the disease.

Within hepatocytes the sporozoites replicate enormously, 40 thousand fold, and differentiate over a week giving rise to a large number of blood stage parasites called merozoites, that are released into the blood stream with the rupture of the infected hepatocyte. In *P. falciparum* infections, once hepatocytes rupture, the liver is cleared of parasites. Another malaria-causing parasite, *P. vivax*, has latent forms, called hypnozoites, that remain in the liver and can be reactivated months after the initial infection (Mueller 2013).

In the blood stream the *P. falciparum* merozoites begin 48 h cycles of invasion of RBCs, replication, RBC rupture and release of merozoites and invasion of RBCs. Parasitemia often exceed densities of 50,000 parasites per microliter of blood. A variety of parasite products are released upon RBC lysis that correlate with the onset of the symptoms of uncomplicated disease: headaches, fever and lethargy. However, what these parasite products are and how they induce malaria symptoms are only poorly understood. At present the parasite antigens and pathogen-associated molecular patterns (PAMPs) that evoke pro-inflammatory responses have not been well characterized. The parasite encodes approximately 5,400 gene products most of which are of unknown function. It is reasonable that some of these products may interact with the host's innate immune system resulting in inflammation and symptoms of malaria. However, to date only a few parasite products have been identified to have such potential including: glycosylphosphatidylinositol (GPI), the lipid that tethers many parasite proteins to the parasite's membrane and binds to a member of the host's innate immune system's pattern recognition Toll-like receptor (TLR) family, TLR2 (Zhu 2005); hemozoin, a detoxified crystalline form of heme produced by the parasite that has been reported to either directly bind to and trigger TLR9 (Coban 2005) or to bind to CpG-containing parasite DNA that binds to and triggers TLR9 (Parroche 2007) and AT-rich motifs in *P. falciparum* DNA that act through unknown immune receptors to induce type 1 IFNs (Sharma 2011). Recently, Unbiased large scale screens for interactions between *P. falciparum* and human proteins have identified additional molecular interactions including one between the *P. falciparum* merozoite surface protein (MSP1) that is shed from the parasite as it enters the RBC and members of the pro-inflammatory protein S100 family (Waisberg 2012). A second interaction was discovered between basigin (CD147) on the RBC surface and the merozoite surface protein *P. falciparum* reticulocyte binding protein homologue 5 (PfRH5) that is critical for invasion (Crosnier 2011). There are a variety of well characterized interactions between parasite ligands and host receptors involved in invasion (Cowman and

Figure 18.1 (Continued) the parasite exports proteins such as PfEMP1s to the RBC surface. PfEMP1s mediate binding of iRBCs to the microvascular endothelium of various tissues **(f)** sequestering the parasites from clearance in the spleen and promoting the inflammation and circulatory obstruction associated with clinical syndromes. In severe disease, such as cerebral malaria, the iRBC sequester in the brain and are associated with microhemorrhages and brain damage **(g).** PfEMP1-mediated rosetting (binding of iRBCs to RBCs) may also contribute to disease **(h).** A small number of blood-stage parasites differentiate into sexual gametocytes **(i)** which are taken up by mosquitoes **(j)** where they differentiate into gametes that fuse to form zygotes, and then develop into motile ookinetes **(k)**. The ookinetes cross the midgut wall and form oocysts **(l)** that develop into sporozoites that enter mosquito salivary glands to complete the life cycle **(a).**

Crabb 2006)but the CD147-PfRH5 interaction is of interest in terms of immune regulation because CD147 is also expressed on the surfaces of a variety of immune cells including DCs, monocytes, T cells and B cells raising the possibility that PfRh5 may modulate the immune function of these cells. Clearly, a better understanding of the parasite products that interact with the host's immune system is needed.

The life cycle of *P. falciparum* in the human host is completed when, in a poorly understood process, the asexual blood-stage parasites differentiate into male and female gametocytes that are taken up in a female mosquito's blood meal. In the mosquito midgut male and female gametes are released and fuse and ultimately differentiate into sporozoites that invade the mosquito's salivary gland, completing the parasite's life cycle.

It is well established that immunity to clinical malaria is ultimately acquired by adolescence such that adults in malaria endemic areas are resistant to the symptoms that accompany blood stage disease. A central question is: What constitutes blood stage immunity? Landmark studies carried out in the 1960's provided evidence that the blood stage disease can be controlled by antibodies. Purified IgG from West African adults passively transferred to children with fever and high parasitemias in West Africa (Cohen 1961), East Africa (McGregor and Carrington 1963) and Thailand (Sabchareon 1991) rapidly and dramatically reduced both the parasite levels and fever. However, we still do not fully understand the specificities of the antibodies that confer protection and the mechanisms by which such antibodies protect in blood stage disease.

In the blood stage there are two obvious antibody targets, namely the infected RBC (iRBC) and the merozoites themselves. There may also be antibody response to gametocytes but these would not impact blood stage disease although such antibodies could block transmission, an area of increasing public health interest (Riley and Stewart 2013). During RBC infection merozoites dramatically remodel the RBC membrane expressing several parasite-encoded proteins on the iRBC surface. Key immune targets among these are the *P. falciparum* encoded membrane protein 1s (PfEMP1s) encoded by the parasite's *var* genes (Miller 2002). PfEMP1s bind to a variety of ligands on endothelia and function to sequester the iRBC in the capillaries of various tissues saving the iRBC from entering the spleen where they would be destroyed. Thus, antibodies specific for PfEMP1s should be highly effective in blocking sequestration and eliminating iRBC and thereby controlling disease. However, the parasite has evolved a remarkable strategy to evade PfEMP1-specific antibody responses. Each parasite clone has approximately 60 *var* genes that encode antigenically distinct PfEMP1 proteins that the parasite expresses clonally, one at a time. However, within a clonal parasite population in an infected individual there is a constant low level of switching to the expression of new *var* genes (Roberts 1992). Antibodies generated in response to the *var* gene expressed by the infecting *P. falciparum* clone block sequestration of the iRBCs and reduce the number of iRBCs and the parasite load (Hommel 1983). The elimination of these iRBC allows the expansion of parasites that have switched to a new *var* gene against which the existing antibodies have little effect. Antibodies are elicited to the new *var* gene product and the process of iRBC elimination and expansion of switched parasites repeats itself over and over. One explanation that has been offered for the slow acquisition of malaria immunity is the time required for an individual to encounter all the *var* genes expressed by parasites in their endemic environment, an explanation supported by several studies as recently reviewed (Portugal 2013). However, other studies suggest that there may be a limited number of PfEMP1s that elicit protective immunity and/or that PfEMP1s contain conserved antigenic determinants with which antibodies cross react. This is a central issue that is important to resolve and will require a detailed characterization of specificities and cross-reactivities of PfEMP1-specific antibodies and their relationship to protection.

In addition to the protective effects of iRBC-specific antibodies on the sequestration of iRBC, antibodies bound to iRBC have the potential to induce the killing or phagocytosis of iRBCs through Fc-receptor- or complement-receptor-mediated activation of NK cells or monocytes. Indeed, it has been observed that antigen-antibody complexes trigger monocytes to release products that are inhibitory to parasite growth in RBCs (Bouharoun-Tayoun 1995). However, such mechanisms have not yet been well characterized.

Merozoites may also be a target of antibody-mediated protection. Theoretically, neutralizing antibodies could be elicited that block the ability of merozoites to invade RBCs. However, *P. falciparum* has multiple functionally redundant receptors to invade RBCs presenting a challenge to the immune system (Cowman and Crabb 2006). In addition, *P. falciparum* merozoite proteins that are necessary for invasion are generally under strong immune selection resulting in a large number of polymorphic forms of these proteins. Nonetheless, *in vitro* parasite growth inhibition assays have identified invasion-blocking antibodies in the serum of individuals living in malaria endemic areas. However, the relationship between invasion blocking and protection from malaria has not been clearly established (Crompton 2010). One recently discovered target for invasion blocking antibodies is PfRH5 (Bustamante 2013; Douglas 2011). In individuals living in malaria endemic areas PfRH5 appears to be less immunogenic relative to other merozoite antigens for reasons that are not clear. However, in animal models PfRH5-containing vaccines have been shown to elicit antibodies that strongly inhibit parasite growth and invasion and are broadly cross-reactive, indicating the feasibility of inducing antibodies that directly block blood stage infections.

The landmark experiment described above showing that malaria-resistant adults have antibodies that can passively transfer protection tells us that some combination of *P. falciparum*-specific antibodies either to the iRBC or to the merozoites can provide immunity to disease. However, at present the specificities of such protective antibodies have not been fully characterized. Identifying antibodies that play a role in protection is an enormous challenge that is complicated by the large number of potential *P. falciparum* antigens encoded in the parasites 5,400 gene genome, and the lack of a clear *in vitro* correlate of immunity. Recently, The use of 'proteome chips' that display the *in vitro* transcribed and translated products of the *P. falciparum* genome have provided a road map for how 'antibody signatures' might be correlated with protection from malaria in population-based studies in endemic areas in African children (Crompton 2010). In addition, new single B cell antibody gene cloning and expression techniques should prove invaluable in understanding the specificity and function of *P. falciparum*-specific antibodies produced in response to *P. falciparum* infection in humans (Tiller 2011). These techniques allow the cloning of genes encoding the variable (V) regions of the heavy (H) and light (L) chain genes expressed by individual antibody producing cells in the peripheral blood of individuals. The cloned V_H/V_L pairs can be expressed in L and H chain vectors and the specificity of the resulting antibodies for *P. falciparum* antigens tested using a *P. falciparum* proteome chip. Analyzing B cells from longitudinal studies in malaria endemic areas in which children are transitioning from a malaria susceptible to a malaria resistant state could provide a wealth of information concerning the V_H and V_L repertoire expressed in response to malaria infections; which V_H and V_L combinations result in malaria-specific antibodies; the degree of somatic hypermutation in the malaria-specific V_H and V_L coding sequences and how the expressed V_H and V_L repertoire changes with repeated infections from childhood to adulthood.

A second key question concerning the acquisition of blood stage immunity is: Why do protective antibodies take so long to develop? At least two non-mutually exclusive possibilities could explain the length of time required to acquire protective immunity in malaria endemic areas as recently reviewed (Portugal 2013). The first is that long times are required for an individual to experience all the clonal variants of *P. falciparum* circulating in a given area. As discussed above, key immune

targets in malaria are the PfEMP1s expressed on iRBC surfaces, however the time required to achieve a protective repertoire of PfEMP1-specific antibodies and the specificities of these antibodies remains controversial.

The second possibility is that the process of antigen activation of B cells to *P. falciparum* is inefficient relative to other pathogens that induce long-lived protective antibodies after one or a few exposures. The generation of an effective antibody memory response is dependent on the acquisition of both memory B cells (MBCs) and long-lived antibody-secreting plasma cells (LLPCs). MBCs have been shown to persist for the lifetime of the individual in several infectious diseases, although the molecular basis of persistence has not been established. MBCs express high affinity, somatically hyper-mutated, isotype-switched antibodies and are responsible for the rapid production of antibody upon reinfection. Several recent studies have shown that *P. falciparum*-specific MBCs are generated in response to natural infection although this process is highly inefficient (Dorfman 2005; Nogaro 2011; Weiss 2010; Wipasa 2010). For example, one longitudinal study in Mali where malaria is transmitted for six months each year showed that the frequency of MBCs specific for two malaria antigens (AMA1 and MSP1) increased incrementally with each malaria season from childhood to adulthood and only approximately half of all adults acquired AMA1- or MSP1-specific MBCs despite years of repeated infections (Weiss 2010). The acquisition of MBCs in malaria is in sharp contrast to that in response to many infections and vaccines. For example, smallpox vaccine-specific MBCs are generated in nearly all individuals following a single vaccination, and these MBCs persist up to 60 years in the absence of re-exposure to the antigen (Amanna 2006).

LLPCs reside in the bone marrow and secrete antibodies that are responsible for the persistent levels of circulating antibody in immune individuals. In longitudinal studies the acquisition of LLPCs, as measured by the surrogate of stable serum antibody levels in the absence of antigen re-exposure, occurs slowly and incrementally over years of exposure to malaria (Crompton 2010). It has been shown that the levels of malaria specific antibodies and not the frequency of MBCs present before infection correlates with subsequent protection from malaria (Crompton 2010). Considering that in blood stage infections, a huge bolus of merozoites enters the circulation at one time and induces symptoms in as little as three days, high levels of pre-existing antibodies may be necessary to control the infection before parasite levels exceed a symptomatic threshold. The MBC response, that produces large increases in the levels of circulating antibodies, peaks 6–10 days after antigen re-exposure that may not be rapid enough to provide control of blood stage infections before symptoms develop.

Taken together, these studies suggest that normal antibody memory is ultimately acquired in adults but that the process is inefficient. However, in malaria there is also recent evidence that B cell differentiation is driven down atypical pathways. In addition to conventional MBCs, individuals living in malaria endemic areas, including in Mali (Portugal 2012; Weiss 2009), Kenya (Illingworth 2013; Muellenbeck 2013), Gabon (Muellenbeck 2013), The Gambia (Nogaro 2011)and Peru (Weiss 2011), have a greatly expanded subpopulation of atypical MBCs (identified by the cell surface markers $CD19^+$ $CD20^+$ $CD21^-$ $CD27^-$ $CD10^-$ $FcRL4^+$). B cells with this phenotype were first described in HIV-infected individuals with high viremia (Moir 2008) and have also been observed in chronic infectious diseases including HCV (Sansonno 2009). For malaria, atypical MBCs appear to be the result of the malaria infection itself, as opposed to other environmental factors, as age matched children living under similar conditions, except for malaria, in rural Kenya showed differential expansion of atypical MBCs (Illingworth 2013). At present the mechanisms at play in the generation of atypical MBCs or their function, if any, in malaria is not known. A recent study using the single B cell V_H/V_L cloning and expression methods described above suggest, based on the V_H and V_L gene usage and V-region somatic hyper-mutational patterns, that conventional and atypical MBCs may arise independently (Muellenbeck 2013).

A key unanswered question is: Why is the acquisition of MBCs and LLPC so slow? At this point in time immunologists' understanding of the cellular and molecular events necessary for the efficient generation of antibody memory both in terms of MBCs and LLPC is incomplete. As progress is made toward an understanding of these events it will be a goal of the malaria immunology field to determine if *P. falciparum* infections and malaria alter the normal course of memory generation and if so how. Nonetheless, there are at present several possibilities worth exploring as recently reviewed (Portugal 2013). For example, the generation of MBCs and LLPC is a T cell-dependent process. The generation of B cell memory occurs in specialized microenvironments in lymphoid tissues called germinal centers and requires the function of $CD4^+$ follicular helper T cells (T_{FH} cells) (Cannons 2013). At present we do not know if malaria infections have an impact on T_{FH} cell development or function. Analysis of T_{FH} cell function in malaria should be facilitated by the recent demonstration that within days following vaccination in humans functional T_{FH} can be identified in the peripheral blood (Streeck 2013). In addition, a variety of $CD4^+$ T cell subpopulations have been described over the last several years that function in antibody responses and in mediating and controlling inflammation as recently reviewed (Finney 2010; Luckheeram 2012; Strutt 2011; Zhu 2010). However, there is not yet a clear picture of how or if these various T cell populations function in malaria. Another possibility that will be discussed below is that anti-inflammatory responses which are evoked to mitigate immune-mediated pathology and likely protect from severe, life-threatening malaria, may impede the efficient acquisition of adaptive immunity that ultimately protects from clinical disease altogether aiding in survival. Clearly, there are many avenues that warrant exploration.

The immune response in severe malaria

Each year there are over 500 million cases of malaria the vast majority of which are uncomplicated and resolve with time even without treatment. However, in a portion of cases, primarily in young children or in older individuals with little or no prior exposure, the infection becomes severe and life threatening resulting in nearly a million deaths each year in Africa alone. Most severe malaria deaths are due to three overlapping clinical syndromes, malaria with impaired consciousness (cerebral malaria), malaria with respiratory distress due to severe metabolic acidosis and severe anemia (Saghafian-Hedengren 2009). One central question is: Why are the majority of children able to resist severe disease, leaving only a few susceptible? Answering this question is complicated by the fact that severe malaria is a complex and, most likely, multifactorial disease and by the fact that there is not a clear picture of the events that initially trigger and then subsequently lead to severe disease. For example, cerebral malaria appears to be the result of a combination of ischemia caused by sequestration of parasites in the brain and microvascular damage, edema, blood brain barrier breakdown, and immune cell activation resulting in inflammation and oxidative stress. Several studies implicate production of the pro-inflammatory cytokines, IFN-γ and tumor necrosis factor (TNF), along with free radicals as causative agents, and the anti-inflammatory cytokines, IL-10 and TGF-β, as protective agents in cerebral malaria (Grau and Craig 2012; Hunt and Grau 2003).

At present we do not have a clear picture of the sequence of events responsible for the progression from mild malaria to severe disease. Several factors in both the human host and the parasite are likely to be involved. In a simple way we can consider that a child could be protected from severe disease at each step in the disease process (Figure 18.2). First, since a child must be infected by *P. falciparum* to develop severe disease, any decrease in parasite transmission by the mosquito vector, due to bed nets or insecticides, for example, will decrease the risk of severe disease. Resistance to infection per se does not appear to be a factor in resistance to severe disease since there is little evidence of acquired sterile immunity to *P. falciparum* infection. In contrast, there are

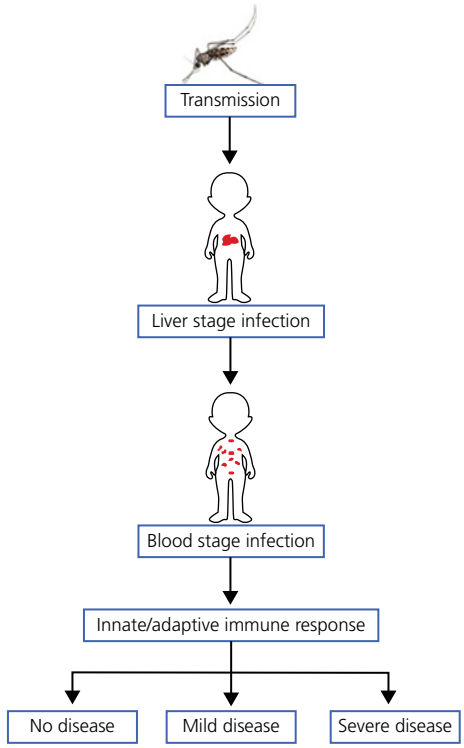

Figure 18.2 Points of control of severe disease. Theoretically, risk of severe malaria could be reduced at several points in the progression of the disease. Transmission can be controlled by mosquito control and bed nets. Infection and the liver stage of the disease do not appear to be points of control in that resistance to liver stage disease is not acquired naturally although future vaccines may produce protection against this stage. The blood stage of the disease can be controlled by anti-malarials and by mutations in a number of genes expressed in the RBC that block invasion or inhibit parasite growth. Once a blood stage infection is established the innate and adaptive immune responses are essential to prevent disease or limit the disease to mild disease.

several known factors that can contribute to decreased risk of severe disease at the blood stage of infection. Obviously, the blood stage of infection can be cured by anti-malarial drugs and intermittent use of anti-malarials in children in endemic areas has been shown to reduce both mild malaria and severe disease (Greenwood 2004; Gosling 2010). In addition, several genetic host factors have been described that reduce risk of severe disease. *P. falciparum* and humans have coevolved (Mu 2002) and the high mortality associated with malaria exerted an enormous selective pressure on the human genome, perhaps a greater impact than any other infectious disease (Kwiatkowski 2005). The best examples of the selective pressure of *P. falciparum* are the number of RBC polymorphisms that occur at relatively high frequency in malaria endemic areas. Sickle cell trait (HbS) is a particularly good example. Remarkably, the HbS allele, that in the homozygous state (HbSS) is lethal in West African children, is nonetheless maintained at a gene frequency of nearly 18% because the heterozygous state (HbAS) confers protection against severe, life-threatening malaria (Molineaux 1985). Calculations of the malaria mortality that is required to maintain this high frequency of HbS in Africa suggest that nearly half of all deaths by any cause in Africa before the introduction of affordable, available anti-malarials were due to malaria (Molineaux 1985).

Studies of the mechanisms underlying protection conferred by HbAS could provide insight into how severe disease is controlled. Indeed, a recent meta-analysis of over 60 studies showed that HbAS conferred significant protection from severe disease but little protection from mild uncomplicated malaria (Taylor 2012). However, thus far, the mechanisms by which HbAS alters the course of *P. falciparum* infections are only incompletely understood and appear to be highly complex and multifactorial, perhaps reflecting eons of genetic interplay between the human host and the *P. falciparum* parasite. As recently reviewed (Bunn 2013), HbAS-mediated protection to malaria has been linked to: enhanced phagocytosis of iRBCs; decreased *P. falciparum* invasion of and growth inside RBCs; aberrant remodeling of the iRBC actin cytoskeleton and abnormal display of the important parasite PfEMP1s on the iRBC surface, decreasing iRBC sequestration in tissues; and inhibition of pathogenic $CD8^+$ T cells. Recently, it was shown that in HbSS and HbAS iRBCs a subset of RBC microRNAs (miRNAs) translocate into the parasite and integrate into essential parasite mRNAs resulting in translational inhibition due to impaired ribosomal loading (LaMonte 2012). This is a remarkable mechanism in itself and the first known anti-pathogen function of a miRNA. It remains a major challenge to determine which, if any, of the phenomenon associated with HbAS are critical for protection from severe disease.

Once a blood stage infection is established a central aspect of protection from progression to severe disease is the immune response. Clearly, features of the parasite also contribute to progression to severe disease, including the PfEMP1 expressed by the parasite that allows sequestration in tissues. One of the best documented examples of the role of PfEMP1s in severe disease is in mature women during pregnancy. Parasites that express one particular PfEMP1, Var2CSA, are able to sequester in the placenta where its ligand, chondroitin sulfate A (CSA) is selectively expressed, causing damage to the placenta, fetus and mother (Fried 1998; Fried and Duffy 1996). In addition, particular PfEMP1s have been recently implicated in parasite sequestration in severe cerebral malaria in children (Avril 2012; Claessens 2012; Lavstsen 2012). Nonetheless, in the absence of an appropriate immune response all malaria infections would likely progress to severe disease.

The question is: Do immune system failures contribute to severe disease? We might consider that failures could occur in either the adaptive or the innate immune response or both. As discussed above, a key role of the adaptive system in controlling malaria is the production of antibodies that control parasite levels. Young children who are most at risk for severe disease are only slowly acquiring antibody immunity that controls blood stage malaria infections. However, if failures of the adaptive immune response contributed significantly to susceptibility to severe disease then uncontrolled parasitemia should accompany severe disease. For cerebral malaria and severe malaria with respiratory distress, that account for nearly all deaths in severe disease, the levels of parasites are not predictive of progression to disease. Of course there are many functions of the adaptive immune response in addition to antibody memory that play roles in protection from severe malaria. Indeed, in mouse models of cerebral malaria $CD8^+$ T cells have been shown to contribute to disease through cytolytic and cytokine mediated mechanisms (deWalick 2007; Engwerda 2002; Nitcheu 2003; Potter 2006). Regulatory T cells have also been implicated in control of severe disease in children (Walther 2009). Defects in such immune mechanism could also contribute to severe disease.

Does the innate system fail in severe disease? It is well appreciated that severe disease has many features of uncontrolled inflammation, and there is evidence that over-vigorous or disordered immune responses are central in severe disease (Langhorne 2008; Riley 2006). Indeed, the pathology of severe disease has been consistently linked to excessive inflammatory responses in children including the production of the pro-inflammatory cytokines TNF-α (Grau 1987; Grau 1989;Kwiatkowski 1990), INF-γ (Grau 1989; Rudin 1997), IL-1β and IL-6 (Grau 1989; Hunt and Grau 2003; Kern 1989) and a linked reduced production of the anti-inflammatory cytokine IL-10

(Day 1999; Kurtzhals 1998; Othoro 1999). Thus, it may be fair to think of severe malaria as the outcome of the failure to control inflammation during a malaria infection. The initiation, propagation and control of inflammation is a complex process involving cells of both the innate and adaptive immune system that can be affected by both host genetic factors and environmental factors. We speculate that both may contribute to severe disease and that children are susceptible to severe disease as a result of inborn errors in immunity coupled with the modulating effect of the child's exposure to pathogens.

Searching for host genes that confer immune resistance to severe malaria

The hypothesis that susceptibility to severe infectious diseases in pediatric populations is due to inborn errors of immunity is gaining experimental support (Alcais 2010). It has been proposed that in populations widely exposed to pathogens from birth, including malaria, life threatening diseases in children, such as severe malaria, result from collections of single-gene variants effecting immunity to primary infection (Alcais 2010). Indeed, Casanova and colleagues modeled malaria mortality with age for simple Mendelian inheritance of genetic susceptibility and concluded that a few dozen gene variants could account for the human genetic contribution to severe malaria (Alcais 2010). The question is: What are these genes?

Although malaria has been recognized to have a strong impact on the human genome due in part to its high level of transmission and high mortality, the full impact of human genetics on resistance to disease remains largely unexplored. Linkage studies have provided evidence for a variety of factors that may influence the severity of malaria, many of which are likely to affect RBC functions necessary for parasite invasion or sequestration of iRBC. One of the best examples of such genes is HbS, as discussed above. Even though HbS and other hemoglobin variants have been strongly selected for by malaria, they are present in only approximately 3–15% of children and cannot explain the resistance of most children to severe disease. In addition there are a number of immune genes that show association with resistance and susceptibility to malaria, including those encoding: MHC molecules that are required for antigen presentation to T cells; FcγRIIB, a potent inhibitory receptor; components of both the type I and type II interferon response that mediate inflammation; IL-12 that controls INF-γ expression and nitric oxide synthesis that generates free radicals (Kwiatkowski 2005). However, few of these associations have been tested in different endemic settings and when tested results have been variable. Thus, further studies will be necessary to definitively identify genes associated with protection in malaria.

The sequencing of the human genome in 2003 allowed genome wide association studies (GWAS) for malaria in which the genomes of patients with severe malaria and of healthy individuals were compared to identify small genetic changes, single-nucleotide polymorphisms, that differed in prevalence between the two groups. The first GWAS study of severe malaria carried out in The Gambia identified only HbS to be associated with resistance (Jallow 2009) even though HbS accounts for only approximately 10% of resistance. The second GWAS study of severe malaria carried out in Ghana identified two novel resistance loci, a RBC calcium pump and an endothelial tight junction protein (Timmann 2012) and confirmed HbS and blood group O as protective. Thus for malaria it would appear that many resistance genes remain to be discovered. Broadly speaking, GWA studies of complex human disease thus far have explained only a small proportion of familial clustering, suggesting that additional strategies will be required to find missing heritability of these diseases (Manolio 2009).

A complementary, hypothesis driven approach to identifying genes that might function in African children to protect from severe disease is to test a specific set of genes that have been characterized to be involved in some aspect of immunity important for malaria resistance, for example the 1,000 or so genes identified to function in innate immunity (de Bakker and Telenti 2010). We hypothesize that genes known to be involved in the control of inflammation may represent such a set worth investigating. In humans such genes have been extensively searched for and studied in systemic autoimmune diseases. In many regards, systemic autoimmune diseases are diseases of uncontrolled inflammation in which both genetic and environmental factors play roles in controlling the onset and severity of the disease. One particularly interesting and highly relevant example of the influences of genetic background on autoimmune disease susceptibility is in systemic lupus erythematosus (SLE) (Deng and Tsao 2010). SLE is a disease characterized by facial rashes, auto-antibodies, deposition of immune complexes in kidneys, inflammation and ultimately multi-organ failure (Rahman and Isenberg 2008; Tsokos 2011). GWAS have rapidly advanced an understanding of the genetic basis of SLE and the involvement of the host immune responses in the disease (Harley 2009). Because SLE is six to eight times more prevalent in women of African descent living outside of Africa than women of European descent, it may be highly relevant to malaria (Molokhia and McKeigue 2006). Indeed, the risk of SLE is directly related to the portion of an individual's genome that is of West African ancestry. Although this observation indicates a genetic basis of SLE susceptibility in African-American women, SLE associations are mostly unexplored in Africans (Harley 2009) and, to date, the West African genes that account for the high risk of SLE in women of African descent have not been identified. Paradoxically, autoimmune disease is strikingly absent from malaria endemic areas of Africa (Greenwood 1968). The low incidence of autoimmune disease in malaria endemic areas led Greenwood and colleagues to speculate in 1970 that malaria suppresses autoimmunity (Greenwood 1970). Nearly 45 years later we speculate that genes selected in West Africa because they are protective against malaria may be susceptibility genes for SLE, out of, but not in malaria endemic areas.

SLE susceptibility and resistance to severe malaria

Is there a relationship between SLE susceptibility genes and resistance to severe malaria? This possibility has been tested in mouse models of SLE and the data thus far demonstrate that SLE susceptibility genes do indeed protect mice against severe cerebral malaria and thus, theoretically, may have been selected for by malaria. In particular, two genetic SLE susceptibility factors were tested in mice for protection from death in severe cerebral malaria caused by *P. berghei* ANKA. C57BL/6 mice infected with *P. berghei* ANKA die within approximately one week with characteristic pathology in the brain including micro-hemorrhages. The first susceptibility factor tested was a deficiency of the inhibitory IgG Fc receptor, FcγRIIb, (FcγRIIb$^{-/-}$ mice), a receptor that plays a central role in the regulation of immune responses. An FcγRIIb deficiency on a C57BL/6 background results in severe lethal autoimmunity by the age of 6–9 months that has many of the hallmarks of human SLE (Bolland 2002). Of interest is the observation that a human allele of FcγRIIb that has a loss of function polymorphism in the transmembrane domain of FcγRIIb (Floto 2005; Li 2003) is significantly more common in Africans (Clatworthy 2007; Willcocks 2010) and in African-Americans (Li 2003) than in Europeans. Recently, Evidence was provided that a loss of function polymorphism in FcγRIIb was associated with protection from severe disease in African children (Willcocks 2010). The second genetic factor tested in mice for protection against cerebral malaria was multiple copies of the RNA sensor Toll-like receptor 7 (TLR7.tg mice) that also results in lethal SLE-like autoimmune disease (Pisitkun 2006).

Remarkably, both FcγRIIb$^{-/-}$ and TLR7.tg mice were protected from cerebral malaria in *P. berghei* ANKA infections (Waisberg 2011). However, neither FcγRIIb$^{-/-}$ mice nor TLR7.tg mice were protected from severe malaria anemia. The impact of SLE-susceptibility was also tested on the outcome of malaria during pregnancy in mice (Waisberg 2013). To do so Mice were infected on day one of pregnancy with the non-lethal parasite *P. chabaudi* and both the course of the disease and the outcome of the pregnancy were followed. FcγRIIb$^{-/-}$ mice appeared to have more severe malaria as compared to wild type mice as judged by increased parasitemia, decreased hematocrit, and greater weight loss. However, SLE susceptibility was protective in that as compared to wild type mice FcγRIIb$^{-/-}$ mice had more total fetuses with similar fetal viability. We concluded from these results that SLE-susceptibility does not appear to have a reproductive cost consistent with the hypothesis that SLE-susceptibility genes were selected for by malaria.

The question becomes: How does genetic susceptibility to SLE protect against severe malaria and can the mechanism of protection provide clues to the paradoxical observation of the extremely low prevalence of autoimmunity in malaria-endemic Africa? The most striking feature of the autoimmune susceptible mice at the time they were infected with *P. berghei* ANKA, at approximately four weeks of age, prior to any overt signs of autoimmunity, is that they were producing more anti-inflammatory cytokines, particularly IL-10, as compared to wild type mice (Waisberg 2011). This observation led to the tentative hypothesis that early in life as the effect of the autoimmune susceptibility genes begin to manifest in inflammation, the immune system counters with an anti-inflammatory response (Figure 18.3). This inflammatory-anti-inflammatory response may teeter-totter back and forth with age with inflammation inevitably winning out. Thus, early in age autoimmune susceptibility genes promote anti-inflammatory responses that protect against cerebral malaria at least in a mouse model. We hypothesize that in young African children autoimmune susceptibility genes create an anti-inflammatory environment that provides protection against severe malaria. Why then do these children not suffer from autoimmune disease as adults as do African American women who carry these genes but live outside of Africa without malaria? One possible answer is because malaria infections that in themselves induce inflammatory responses also provoke anti-inflammatory responses that protect from autoimmune disease. Is there evidence that malaria infections induce anti-inflammatory responses that control inflammation (Figure 18.4)? One of the first indications that this might be the case came from studies of experimental infections of individuals with tertiary syphilis with *P. falciparum* exploring the possibility that malaria-induced fevers may be therapeutic for syphilis (Collins and Jeffery 1999). Patients were given an initial *P. falciparum* infection, drug cured when parasitemias reached high levels and then days later infected a second time. Remarkably, fever, as a surrogate for inflammation, reaches higher levels at lower parasitemias in the first as compared to the second infection suggesting that the first infection induced an anti-inflammatory response that controlled inflammation during the second infection. Evidence has also been provided for malaria-induced tolerance to inflammation. Individuals who were experimentally infected with *Plasmodium* parasites and developed malaria had a decreased febrile response to endotoxin as compared to infected individuals who did not develop malaria (Rubenstein 1965). In a mouse model of malaria evidence was provided that dendritic cells (DCs) taken from mice prior to a malaria infection produced proinflammatory cytokines when stimulated *in vitro* with TLR agonists (Perry 2005). However, DCs from mice immediately after an infection produced anti-inflammatory cytokines in response to TLR agonists *in vitro*. Thus, in response to the inflammation induced by malaria the mice mounted an anti-inflammatory response. In a recent unpublished study, Crompton and colleagues provided evidence of a similar phenomenon in children. Immediately before their first case of febrile malaria at the beginning of a six-month malaria transmission season in Mali, children's PBMCs had a pro-inflammatory phenotype in response to stimulation with parasite lysates *in vitro*. Seven days after treatment of febrile malaria with anti-malarials, the children's PBMCs produced significantly higher levels of IL-10 and down regulated the expression of pro-inflammatory

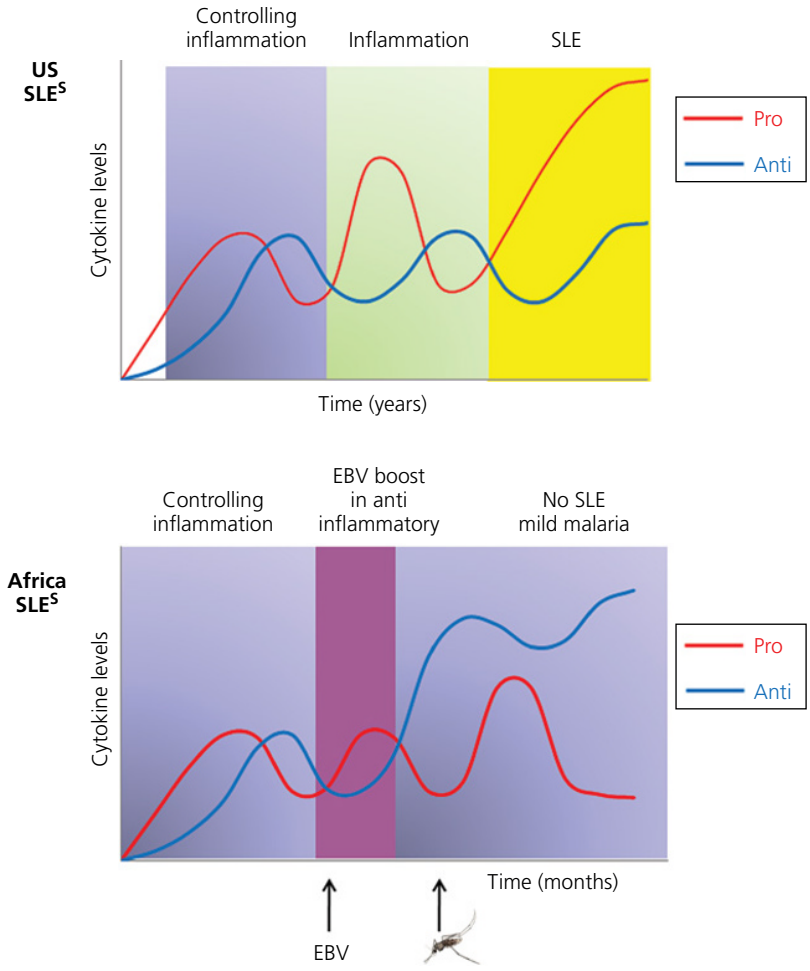

Figure 18.3 Selecting for and controlling genetic susceptibility to autoimmunity in malaria endemic areas. We propose that genetic susceptibility to the autoimmune disease, SLE, may have been selected for in Africa as it protects from severe disease. **Top.** In the US, SLE^s genes would initiate inflammation that would trigger an anti-inflammatory response. As the anti-inflammatory response wanes the uncontrolled SLE^s-mediated inflammation would rise again. Ultimately, the balance would tip toward inflammation and autoimmune disease. **Bottom.** In Africa SLE^s genes have the same potential to cause inflammatory autoimmunity. However, this potential is attenuated by environmental pathogens, *P. falciparum* infections themselves or perhaps viral infections such as EBV resulting in an anti-inflammatory environment that protects against both severe disease and autoimmunity.

cytokines and chemokines *in vitro* in response to parasite lysates. Thus, malaria infections left these children in an anti-inflammatory state, at least for a period of time. Such an anti-inflammatory response may explain a puzzling finding that even in areas of extremely high malaria transmission where children may receive hundreds of bites from infected mosquitoes, susceptible children experience on average only two episodes of malaria each season (Crompton 2008).

To close the loop we might ask: Is there evidence that the immune response to malaria is protective against SLE? In 1970, the observation that a number of immunological changes occur in a large portion of otherwise healthy individuals living in malaria endemic Africa coupled with the rarity of autoimmune disease in African led Greenwood and colleagues to propose that parasite infections

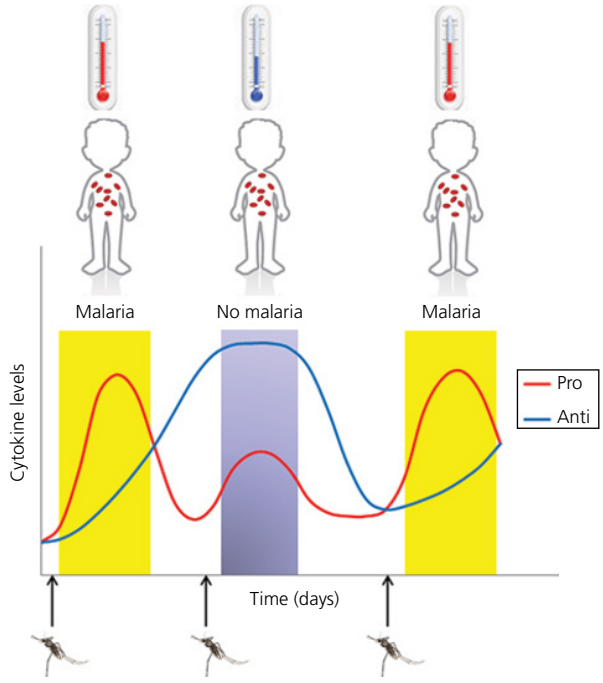

Figure 18.4 Pro- and anti-inflammatory battles in malaria. Blood stage infections induce inflammatory responses producing the symptoms of mild malaria. To control the inflammatory response and avoid damage, the immune system mounts an anti-inflammatory response. Malaria symptoms during the anti-inflammatory response may be attenuated producing asymptomatic infections. As the anti-inflammatory response wanes new *P. falciparum* infections produce inflammation and disease. We propose that the anti-inflammatory response although protective may hinder the acquisition of MBC and LLPCs.

can suppress autoimmune disease (Greenwood 1970). In a mouse model of SLE, Greenwood *et al.* (Greenwood 1970) demonstrated that this was indeed the case, showing that infection with *P. berghei yoelii* was associated with protection against autoimmune disease in NZB and (NZB × NZW)F, mice. Thus, we speculate that African children who carry autoimmune susceptibility genes do not as adults suffer autoimmune disease as they benefit from the anti-inflammatory mechanisms initiated spontaneously by the autoimmune susceptibility genes and their amplification by malaria infections and potentially other pathogens that are common in malaria endemic areas (Figure 18.3). In areas of the world where malaria has been eliminated or never existed, the same autoimmune susceptibility genes ultimately win out producing life-threatening autoimmunity.

The relationship between the pathogen environment and susceptibility to severe malaria

The observation that malaria infections have the potential to protect from autoimmune disease raises the question: Is the effect of malaria infections on the development of autoimmune disease unique? The answer, at least from mouse models, appears to be no. Infections of mice with gamma herpes virus, a mouse model for human EBV-infections, were recently shown to protect against SLE (Larson 2012). If herpes infections can protect against autoimmunity, is it possible that human

herpes virus infections protect against severe malaria in children? EBV infections are ubiquitous world-wide but the age at which children become infected varies geographically. In Africa nearly 80% of children become infected by 25 months of age as evidenced by the appearance of EBV-specific antibodies in the blood in contrast to the U.S. where only 50% of children are infected by age 5 (Biggar 1978; Cannon 2010). EBV established latent infections that appear to promote an anti-inflammatory environment. Latently infected children have markedly lower levels of circulating pro-inflammatory cytokines including IFNγ (Saghafian-Hedengren 2009). During latency EBV infected cells produce the EBV gene product BCRF1, a homologue, of IL-10, an anti-inflammatory cytokine that inhibits IFNγ production and reduces the generation of superoxide anions by PBMCs and monocytes (Niiro 1992). Moreover, EBV-infected lymphoblastoid cell lines produce large quantities of IL-10. It was shown in a mouse model of uncomplicated malaria that latent infection with the EBV-like mouse gamma-herpes virus was protective, resulting in a significant reduction in parasitemia as compared to malaria infection alone (Haque 2004). In addition, EBV particles are capable of binding to platelets and *in vitro* induced the release of TGF-β (Ahmad and Menezes 1997), a potent immunosuppressive agent that plays an important role in preventing severe tissue pathology in mouse malaria and is associated with a reduced risk of the severity of malaria infection (Esamai 2003; Omer 2003; Omer and Riley 1998).

Taken together we make the highly speculative proposal that the combination of the action of autoimmunity susceptibility genes and impact of early malaria or EBV infections bias the immune systems of African children toward anti-inflammatory responses that protect from severe malaria. We predict that autoimmune susceptibility genes and early susceptibility to herpes virus infections are in a somewhat counter intuitive way protective against highly inflammatory severe malaria.

Summary

In this review we contrasted the acquisition of immunity to uncomplicated malaria and to severe disease. We pointed out that one of the most striking features of the acquisition of immunity to uncomplicated disease is the years of exposure to parasites that is required to achieve protection. In contrast, protection from severe malaria is acquired relatively quickly, possibly after a single infection. As the hallmark of severe disease is uncontrolled inflammation, we concluded that immunity to severe disease must involve the control of inflammation. We noted that all young children are susceptible to *P. falciparum* infections and to uncomplicated disease but that only a minority of children appears susceptible to severe disease. We speculated that susceptibility to severe disease will depend on the genetic composition of the child and their pathogen exposure. We suggested that important resistance genes may be those that are associated with susceptibility to autoimmune disease and that pathogens of interest may be virus in the herpes family. Lastly, we concluded that the effort to control inflammation during malaria, while essential to survival, may be deleterious to the acquisition of adaptive malaria immunity (e.g. antibodies). Thus, the slow acquisition of antibody-mediated protection from uncomplicated malaria and control of inflammation necessary for protection from severe malaria may be two sides of the same coin.

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CHAPTER 19

Progress in development of malaria vaccines

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A number of highly effective vaccines that are routinely used in large-scale immunization programs and provide enormous public health benefits have been successfully developed over the past century. These vaccines primarily target viral or bacterial pathogens that elicit protective immunity following natural infection. Development of vaccines against pathogens that cause chronic infections and do not elicit sterilizing protective immunity is more challenging. Such pathogens include viruses such as human immunodeficiency virus (HIV), bacterial pathogens such as *Mycobacterium tuberculosis*, and parasites such as *Plasmodium falciparum* and *Plasmodium vivax*, which cause malaria. Here, we review the current status of efforts to develop vaccines against malaria parasites and discuss the challenges and potential ways forward.

Despite recent progress in reducing the malaria burden following intensified malaria control efforts, malaria remains a significant public health problem in many regions of the tropical world. It is estimated that there were 207 million clinical malaria cases leading to 627,000 deaths in 2010 (World Health Organization 2012). However, other studies have suggested that the death toll due to malaria could be significantly higher, exceeding 1.2 million in 2010 (Murray 2012).

Mortality due to malaria is primarily attributed to *P. falciparum* infections and occurs mainly in children residing in malaria-endemic regions of Africa. Pregnant women are also uniquely susceptible to severe disease following infection with *P. falciparum*, which can put the lives of both the pregnant mother and child at risk. Outside Africa, *P. vivax* is responsible for about 80 million cases of malaria and is widespread in tropical regions of South and Southeast Asia, Pacific Islands, and Latin America (Mendis 2001).

Historically, *P. vivax* was thought to cause only mild malaria, with *P. falciparum* primarily being responsible for severe malaria and malaria-related mortality. However, more recently, clinical epidemiology studies have demonstrated that *P. vivax* can also lead to severe malaria syndromes and cause death (Kochar 2007, 2009; Tjitra 2008; Alexandre 2010; Costa 2012). It is thus important to develop vaccines to protect against both *P. falciparum* and *P. vivax* malaria.

Immunity to malaria

Following repeated exposure to *P. falciparum* infection over several years, residents of malaria-endemic areas develop protective immunity that reduces the frequency of malaria episodes and provides protection against severe malaria and malaria-related death (Riley and Stewart 2013). Acquisition of immunity following repeated natural exposure to *P. falciparum* infections is associated

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with reduced parasite densities, suggesting that immunity may include an anti-parasite component. However, sterilizing immunity against *P. falciparum* is never achieved. As a result, it is primarily infants and children younger than 5 years who suffer multiple *P. falciparum* malaria episodes and are at significantly higher risk of progressing to severe malaria and death in endemic areas compared to older children or adults, who have acquired protective immunity following repeated infection.

Naturally acquired immunity to *P. vivax*, which reduces the frequency of *P. vivax* malaria episodes with age, develops more rapidly compared to *P. falciparum* in endemic populations (Mueller 2013). As in the case of *P. falciparum*, sterilizing immunity against *P. vivax* infection is also never acquired. The observations that repeated exposure to *P. falciparum* and *P. vivax* infections leads to acquisition of immunity suggest that it should be possible to elicit protective immune responses using vaccines. Moreover, transfer of immunoglobulins from immune adults from Africa to non-immune adults in Southeast Asia who were infected with *P. falciparum* has been shown to clear-blood stage parasites, demonstrating that antibodies play a role in parasite clearance (Cohen 1961). The targets of such protective antibodies are, however, not known, and the correlates of protective immunity remain to be defined.

Traditional approaches to develop vaccines against infectious agents have commonly used attenuated pathogens for immunization. In case of malaria, immunization of mice with repeated bites of irradiated *Plasmodium berghei* sporozoite–infected *Anopheles* mosquitoes provides protection against subsequent challenge with *P. berghei* sporozoites (Nussenzweig 1967). Similarly, immunization of humans with bites of irradiated *P. falciparum* sporozoite–infected *Anopheles* mosquitoes protects against *P. falciparum* sporozoite challenge (Clyde 1973; Clyde 1975, 1990). Inoculation of volunteers with *P. falciparum* sporozoites by infected mosquito bites three times at 28-day intervals during which they were administered chloroquine also resulted in acquisition of sterilizing protective immunity against challenge with *P. falciparum* sporozoites (Bijker 2013). These studies demonstrate that it is possible to elicit protective immune responses against *P. falciparum* pre-erythrocytic stages. Understanding the correlates of such protective immune responses could inform approaches for development of malaria vaccines.

Life cycle of malaria parasites and points of intervention with vaccines

Malaria parasites have a complex life cycle. Infection in humans commences with the bite of an infected female *Anopheles* mosquito, which injects a small number of *Plasmodium* sporozoites (<100) in the human host during a blood meal. The sporozoites traverse tissues and enter blood vessels that carry them through circulation to the liver, where they invade hepatocytes. Following invasion, *Plasmodium* sporozoites multiply around 10,000-fold by schizogony and differentiate into merozoites over a period of around 10 days. Merozoites emerge from infected hepatocytes packaged in membrane-bound structures called merosomes. Once released from merosomes into the blood stream, merozoites go on to invade and multiply within host erythrocytes. Repeated cycles of invasion, multiplication and egress lead to a rise in blood-stage parasitemia (~10–20-fold increase in parasitemia per cycle).

The blood stage of the life cycle is responsible for all the clinical symptoms of malaria. The virulence of *P. falciparum* is partly attributed to the ability of blood-stage trophozoites and schizonts to sequester in the vasculature of diverse host organs. Sequestration is mediated by variant surface antigens belonging to the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) family, which are expressed on the surface of *P. falciparum*–infected erythrocytes (Miller 1994). Sequestration in host organs enables *P. falciparum* to escape spleen-dependent clearance mechanisms. Sequestration

in capillaries and venules of the brain is implicated in the severe malaria syndrome of cerebral malaria. In the case of *P. vivax*, some infected hepatocytes differentiate into hypnozoites that can remain dormant for weeks to years before reactivation produces merozoites that establish a blood-stage infection, causing malaria. *P. vivax* infection can thus lead to relapse of malaria symptoms months or years after infection.

During the blood stage, some parasites differentiate into male and female gametocytes that are taken up by mosquitoes during a blood meal. The male and female gametocytes fertilize in the mosquito midgut and develop into a small number of ookinetes (<10), which cross the midgut epithelium to form oocysts on the outer midgut wall. Thousands of sporozoites develop in each oocyst and are released into the hemocele upon maturation. The sporozoites migrate toward and invade salivary glands to complete the parasite life cycle.

The parasite life cycle provides multiple points for immune intervention (Figure 19.1). Antibodies directed against sporozoite surface proteins can inhibit hepatocyte invasion, whereas cell-mediated immune responses ($CD4^+$ and $CD8^+$ T cells) can target and clear infected hepatocytes. Pre-erythocytic vaccines that target sporozoites or infected hepatocytes can prevent infection and appearance of

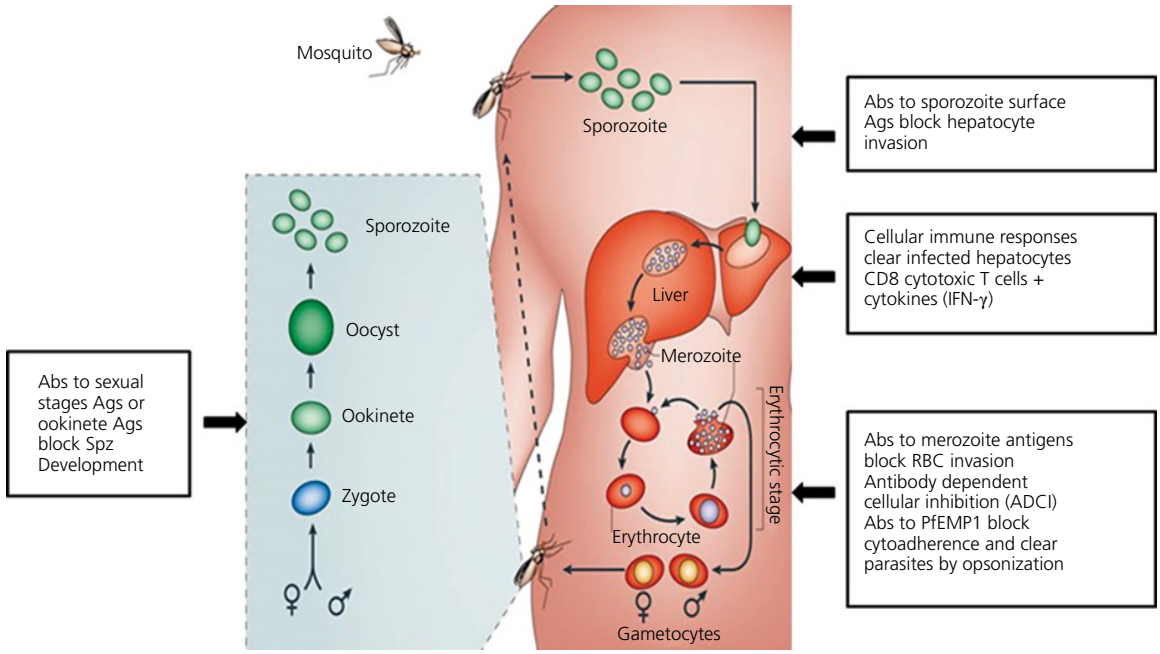

Figure 19.1 Immune mechanisms can target malaria parasites at different stages in the life cycle. Antibodies (Abs) elicited against sporozoite surface antigens (Ags) may target sporozoites and block hepatocyte invasion. Cellular immune responses including cytotoxic CD8 T-cell responses may clear infected hepatocytes and prevent emergence of merozoites. Antibodies against merozoite Ags may block erythrocyte invasion by blocking receptor–ligand interactions that mediate invasion or by antibody-dependent cellular inhibition (ADCI). Antibodies against variant surface antigens expressed on the surface of *P. falciparum*–infected trophozoites and schizonts may block cytoadherence and mediate clearance by alternative mechanisms such as antibody-dependent opsonization. Antibodies against sexual or transmission stages will block transmission from infected to uninfected individuals. *Source:* Dondorp AM, Yeung S, White L, Nguon C, Day NPJ, Socheat D, von Seidlein L. 2010. Artemisinin resistance: current status and scenarios for containment. *Nature Reviews Microbiology.* 8:272–280.

blood stage parasites if they are 100% effective. Partially effective pre-erythrocytic vaccines will reduce the number of merozoites released and might lead to delayed appearance of blood-stage parasites following infection, decreasing morbidity. Antibodies directed against merozoite antigens can either inhibit erythrocyte invasion directly (Crompton 2010) or mediate clearance of blood stage parasites by antibody-dependent cellular inhibition mechanisms mediated by monocytes or macrophages (Druilhe and Perignon 1994; Bouharoun-Tayoun 1995). Finally, antibodies against transmission stages can limit fertilization and development of stages in the mosquito, preventing transmission (Lavazec 2008).

Malaria vaccine development efforts have primarily focused on providing protection against *P. falciparum* malaria due to the significant mortality associated with *P. falciparum* infections. However, given the renewed call for eradication of malaria, it is necessary to consider what role malaria vaccines could play in such efforts. Malaria vaccines that reduce transmission efficiency could, in conjunction with other control tools such as insecticide-treated nets, indoor residual spraying, and early detection and treatment, greatly aid efforts to eliminate malaria from defined endemic regions, eventually leading to malaria eradication. Such vaccines that interrupt malaria transmission could target sexual- or mosquito-stage antigens to block development of next-generation sporozoites in the mosquito, or vaccines could be based on highly effective pre-erythrocytic– or erythrocytic-stage antigens that drastically reduce blood-stage parasitemia, leading to an impact on malaria transmission efficiency at an individual level and reduction of transmission intensity at a community level. Development of a highly efficacious malaria vaccine that both protects against clinical malaria and prevents transmission is most attractive and will likely require combination of multiple antigens from different stages.

Pre-erythrocytic stage vaccines

The most advanced malaria vaccine under development, RTS,S, is based on the *P. falciparum* circumsporozoite protein (PfCSP), the major surface protein on sporozoites. PfCSP contains a central tetrapeptide repeat region and C-terminal conserved thrombospondin (TSP)-like region that mediates binding to hepatocytes. The central repeat region, which contains tetrapeptide repeats that serve as B cell epitopes, elicits strong antibody responses. The TSP-like region contains variant $CD4^+$ and $CD8^+$ T cell epitopes. RTS,S is composed of hepatitis B surface antigen (HBsAg) particles with around a quarter of the HBsAg molecules fused to a truncated central repeat region and C-terminal TSP-like region of PfCSP (Casares 2010).

Evaluation of the efficacy of RTS,S in a series of challenge trials in malaria-naïve adults demonstrated that immunization with RTS,S formulated with adjuvants AS01B and AS02A, which contain monophosphoryl lipid A (MPL, a Toll-like receptor 4 [TLR4] agonist) and the saponin QS21, protects 30% to 50% of immunized volunteers against challenge by mosquitoes infected with a homologous *P. falciparum* strain (Casares 2010). Protection against *P. falciparum* sporozoite challenge appears to be associated with antibody titers and $CD4^+$ cellular immune responses against PfCSP, although the association with $CD4^+$ responses is weaker compared to antibody responses (Kester 2009; Olotu 2010).

Phase II trials conducted in semi-immune African adults yielded only short-term protection against natural *P. falciparum* infection in about 35% of immunized volunteers in The Gambia (Bojang 2001), and no statistically significant protection was observed in Kenya (Polhemus 2009). However, evaluation of efficacy of RTS,S against natural *P. falciparum* infection in the first pediatric Phase II field trials in endemic regions of Africa yielded promising results. Alonso and colleagues demonstrated in a placebo-controlled efficacy trial conducted in 1- to 4-year-old children residing

in a malaria-endemic region of Mozambique that RTS,S formulated with AS02A yields a protective efficacy of 29% against first clinical malaria episode and efficacy of around 58% against severe malaria (Alonso 2004). Similar levels of protective efficacy ranging from 30–50% were observed in Phase II trials conducted subsequently in children and infants in malaria-endemic regions of Mozambique, Tanzania, and Kenya (Alonso 2005; Abdulla 2008; Bejon 2008; Sacarlal 2009). Anti-PfCSP antibody titers were associated with protection against *P. falciparum* infection but not with protection against clinical *P. falciparum* malaria in these studies (Aponte 2007; Bejon 2008; Guinovart 2009).

Following these promising results, a large multicenter Phase III field trial was undertaken in around 15,000 infants in two age groups, 6 to 12 weeks of age and 5 to 17 months of age, at different malaria-endemic locations in 7 sub-Saharan African countries. Interim data analysis for prevention of first or only episode of malaria in 6000 children in the 5- to 17-month age group over a 12-month follow-up period after last vaccination showed vaccine efficacy of 55.8% and efficacy against severe disease of 47.3% (Agnandji 2011). A second interim data analysis reported efficacy for the prevention of first or only malaria episode of malaria in 6- to 12-week-old infants during a 12-month follow-up period after last vaccination to be 31.3% and efficacy against severe malaria to be 36.6% (Agnandji 2012). Available safety data indicates that RTS,S is safe and well tolerated in infants and children (Agnandji 2011; Agnandji 2012).

Final results of the Phase III clinical trial indicate that RTS,S elicited 43.9% protection against clinical disease over a 32-month follow-up period in 5- to 17-month-old children and lower protection of 27.8% in younger infants (RTS,S Clinical Trial Partnership 2015). For the same follow-up period, a four-dose schedule achieved protection against severe disease of around 32.2% in 5- to 17-month-old children, with no protection against severe malaria in infants (RTS,S Clinical Trial Partnership 2015). Furthermore, there was no statistically significant protection against severe disease in 5- to 17-month-old children for a three-dose schedule. Given the limited protective efficacy achieved and uncertainty in reaching the four doses required in older children to achieve protection against severe disease, WHO recommended further studies of RTS,S in three to five malaria-endemic African settings to generate further data on potential impact on mortality and assess the ability of health systems to deliver the vaccine. Further studies are needed to understand the reasons for the differences observed in efficacy of RTS,S in different age groups. Moreover, a better understanding of the immune correlates associated with protective efficacy is needed. This may allow the development of strategies to augment the observed efficacy of RTS,S and improve efficacy.

Approaches to improve efficacy of RTS,S need to be explored. One approach is to combine RTS,S with other liver-stage antigens that yield partial efficacy. Another approach to improve efficacy is to use alternative methods such as viral vectors to deliver PfCSP and other liver-stage antigens in an effort to achieve higher-titer antibody responses as well as stronger $CD4^+$ T-cell responses. Such alternative delivery approaches may also allow development of effective $CD8^+$ cytotoxic T cell responses, which have been shown to mediate protection against pre-erythrocytic stages in animal models. For example, use of recombinant human adenovirus 35 expressing PfCSP (Ad35-PfCSP) for priming, followed by boosting with RTS,S, significantly enhanced $CD4^+$ T cell responses to PfCSP in rhesus monkeys (Stewart 2007). Alternative delivery systems based on fowlpox virus, chimpanzee adenovirus (ChAd), and modified vaccinia virus (MVA) vectors are also being used to develop prime-boost strategies to elicit potent humoral and cellular immune responses (Hill 2010; O'Hara 2012). Chimpanzee adenovirus–based vectors avoid the problems of preexisting immunity to human adenoviral vectors, and use of such heterologous prime-boost approaches yields significantly higher cellular immune responses compared to single vector immunization.

Other pre-erythrocytic antigens that are under development as vaccine candidates include thrombospondin-related adhesive protein (PfTRAP) and cell traversal protein for ookinetes and sporozoites (PfCelTos). PfTRAP plays a functional role in sporozoite motility and attachment to host hepatocytes to mediate invasion (Sultan 1997), whereas PfCelTos is used by sporozoites and ookinetes for traversal across host cells (Kariu 2006). Priming with ChAd63 expressing ME-TRAP, which is composed of a peptide from TRAP fused to multiple CD4, CD8 and B cell epitopes from diverse liver and blood stage antigens, followed by boosting with MVA expressing ME-TRAP, elicited significant $CD8^+$ T-cell responses (Ogwang 2013) and elicited partial protection against challenge with heterologous sporozoites (Ewer 2013). Protection was associated with presence of interferon-γ (IFN-γ)–secreting $CD8^+$ T- cells (Ewer 2013). Identification of additional pre-erythrocytic antigens with protective B- and T-cell epitopes and optimization of delivery methods for such novel antigens may help augment protective immune responses, leading to significantly higher protective efficacy than has been achieved so far with RTS,S.

Blood-stage vaccines

All the clinical symptoms of malaria are attributed to the blood stage of the parasite life cycle. During the blood stage, *Plasmodium* merozoites invade and multiply within erythrocytes. Antibodies against merozoite antigens that play a role in erythrocyte invasion are likely to inhibit invasion and block blood-stage parasite growth (Yazdani 2006a). Monoclonal antibodies (MAbs) raised against *P. falciparum* merozoites that blocked erythrocyte invasion were used to identify a 195-kDa protein on the merozoite surface referred to as merozoite surface protein-1 (PfMSP1). Homologues of PfMSP1 are found on the surface of merozoites of all *Plasmodium* species, suggesting that it plays a functionally essential role. PfMSP1 is proteolytically processed into 83-kDa, 30-kDa, 38-kDa, and 42-kDa fragments that form a complex on the merozoite surface (Blackman 1991a; Blackman and Holder 1992). A final proteolytic step cleaves the 42-kDa fragment, leaving a C-terminal 19-kDa fragment ($PfMSP1_{19}$) on the merozoite surface following invasion (Blackman 1991b; Harris 2005).

$PfMSP1_{19}$, which shares homology with epidermal growth factor (EGF)-like domains, is attached to the merozoite surface by a glycophosphadityl inositol (GPI) anchor at the C-terminus (Blackman 1991a). Antibodies raised against $PfMSP1_{19}$ inhibit erythrocyte invasion by *P. falciparum* merozoites *in vitro* (Moss 2012). Moreover, immunization of mice and nonhuman primates with recombinant *Plasmodium yoelii* $MSP1_{19}$ and *Plasmodium cynomolgi* $MSP1_{19}$ formulated with Freund's adjuvant provides protection against *P. yoelii* and *P. cynomolgi* challenge, respectively (Hirunpetcharat 1997; Perera 1998). Immunization of Aotus monkeys with the 42-kDa C-terminal fragment of PfMSP1 ($PfMSP1_{42}$) formulated with complete and incomplete Freund's adjuvant, Montanide ISA720, and AS02A elicited partial protection against blood-stage challenge with homologous *P. falciparum* strain (Singh 2006a; Lyon 2008). Protection depended on the adjuvant used and was associated with ELISA titers against $PfMSP1_{42}$ and inhibitory activity against *in vitro* parasite growth (Singh 2006a; Lyon 2008).

Sequence analysis of the C-terminal $PfMSP1_{42}$ region from diverse *P. falciparum* field isolates has identified presence of dimorphic alleles referred to as the 3D7 and MAD20 alleles (Tanabe 1987). Moreover, point mutations are reported in the amino acid sequence of $PfMSP1_{19}$. Field studies in malaria-endemic regions have demonstrated that acquisition of antibodies against $PfMSP1_{19}$ is associated with protection against *P. falciparum* malaria (Egan 1996; Stanisic 2009; Dent 2012). Based on these observations, PfMSP1 was regarded as one of the most promising subunit vaccine candidates for *P. falciparum* malaria.

The efficacy of a vaccine candidate based on *P. falciparum* 3D7 allele of $PfMSP1_{42}$ formulated with AS02A has been tested in children residing in *P. falciparum*–endemic areas in Africa. Recombinant $PfMSP1_{42}$ was immunogenic and yielded specific antibodies that reacted with the native antigen as determined by immunofluorescence assays (IFA) with *P. falciparum* schizonts. However, immunization with recombinant $PfMSP1_{42}$ (3D7 allele) formulated with AS02A did not elicit any protection against *P. falciparum* malaria (Ogutu 2009). The lack of protection is likely due to the inability of the vaccine to raise antibodies with sufficiently high invasion-inhibitory titers and the presence of polymorphisms in the target antigen sequence in field isolates.

Sequence analysis of PfMSP1 alleles of *P. falciparum* strains circulating in clinical trial sites in East as well as West Africa indicated that only a minor fraction had the 3D7 $PfMSP1_{42}$ allele. The FVO and CAMP $PfMSP1_{42}$ alleles had higher prevalence in both East and West Africa (Otsyula 2013). Immunization of volunteers with $PfMSP1_{42}$ FVO allele formulated with AS01 elicited high-titer antibodies that recognized both the homologous FVO allele and heterologous CAMP and 3D7 alleles by ELISA (Otsyula 2013). However, antibodies exhibited poor growth-inhibitory activity (GIA) even against the homologous *P. falciparum* FVO strain (Otsyula 2013). Alternative delivery methods such as prime-boost strategies using viral vectors or virus-like-particles may be needed to achieve high-titer functional antibodies against PfMSP1 that block parasite growth efficiently to achieve efficacy. Other MSPs such as MSP-2 and MSP-3 have also been evaluated as candidate vaccine antigens in human trials and are discussed later.

Another leading *P. falciparum* malaria vaccine candidate, apical merozoite antigen-1 (PfAMA-1), was also identified based on invasion-inhibitory MAbs raised against *P. falciparum* merozoites (Deans 1982, 1984; Peterson 1989). PfAMA-1, which is localized in micronemes and is translocated to the surface when merozoites are released following schizont rupture (Healer 2002; Singh 2010), is thought to play a central role in formation of the junction between the invading merozoite and target erythrocyte during invasion (Srinivasan 2011, 2013), although genetic data suggest that its role might not be essential (Giovannini 2011; Bargieri 2013). PfAMA-1 binds to a rhoptry neck protein (PfRON2), which is secreted from the rhoptries and gets inserted into the target erythrocyte membrane (Srinivasan 2011). Inhibition of this interaction inhibits invasion (Srinavasan 2013). Immunization of laboratory animals with recombinant PfAMA-1 elicits high-titer invasion-inhibitory antibodies, supporting its use as a recombinant malaria vaccine.

Immunization with a combination of PfAMA-1 and the PfRON2 peptide that form a complex elicits highly potent invasion-inhibitory antibodies (Srinivasan 2014). However, PfAMA-1 is highly polymorphic, and the invasion-inhibitory activity of anti-PfAMA-1 sera is allele-specific (Marshall 1996; Escalante 2001; Takala 2009). Immunization of children in an endemic region of Africa with PfAMA-1 failed to provide any protection against *P. falciparum* malaria (Sagara 2009). However, there was significant reduction in the incidence of *P. falciparum* malaria cases attributed to the homologous vaccine-specific PfAMA-1 allele, suggesting that the antibodies elicited are allele-specific (Thera 2011).

These results highlight the problem of sequence polymorphisms in target antigens for development of efficacious vaccines for malaria. One strategy to overcome the diversity problem is development of recombinant PfAMA-1 variants that cover most PfAMA-1 antigenic polymorphisms and that elicit strain-transcending invasion-inhibitory antibodies when used for co-immunization (Remarque 2008). Two studies have also attempted to address this problem by immunizing with a combination of different AMA-1 allelic proteins representing multiple *P. falciparum* strains (Miura 2013a; Dutta 2013). It has been demonstrated that antibodies against four AMA-1 allelic forms termed Quadvax are sufficient to produce potent cross-strain–transcending invasion-inhibitory antibodies (Dutta 2013). AMA-1 has a crucial role in *P. falciparum* erythrocyte invasion and has evolved a high level

of antigenic polymorphisms to evade the immune system. These studies have shown novel approaches to circumventing the problem of antigenic polymorphisms.

The invasion of erythrocytes by *Plasmodium* merozoites is mediated by multiple receptor–ligand interactions, as described in Chapter 3. Parasite ligands that mediate interactions with host receptors during invasion include two families of erythrocyte-binding proteins known as the DBL (Duffy binding-like) family and RBL (reticulocyte binding-like) family. The DBLs include the 175-kDa *P. falciparum* erythrocyte binding antigen (PfEBA175), its homologues (EBA-140, EBA-181, EBL-1), and *P. vivax* Duffy binding protein (PvDBP), *P. knowlesi* Duffy binding protein (PkDBP), and *P. knowlesi* β and γ proteins that bind receptors other than the Duffy antigen on rhesus erythrocytes (Adams 1992).

DBLs are localized in micronemes and are released to the merozoite surface during invasion. Interaction with the Duffy blood group antigen was considered essential for junction formation during invasion of human erythrocytes by *P. vivax* and *P. knowlesi* (Miller 1975; Miller 1976; Singh 2005); however, reports have observed *P. vivax* infections in Duffy-negative individuals (Menard 2010), suggesting that the Duffy interaction is no longer crucial for *P. vivax* invasion.

Although there is no direct evidence that DBLs are present at the junction, it is clear that interaction of the DBLs with their receptors is critical for facilitating junction formation during invasion. For example, the interaction of EBA175 with its receptor, glycophorin A, has been shown to trigger rhoptry secretion (Singh 2010), which may contain components required for junction formation. In the absence of interaction of DBLs with their receptors, rhoptry proteins may not be released, preventing junction formation. Antibodies against DBLs may also block receptor–ligand interactions required for junction formation, leading to inhibition of red cell invasion and parasite growth.

The receptor-binding domains of DBLs were mapped to a conserved N-terminal cysteine-rich region, region II (PvDBPII), which is characteristically referred to as the DBL domain after the first binding domain identified from PvDBP (Chitnis and Miller 1994; Sim 1994). Methods to produce PvDBPII in its correctly folded functional conformation have been developed (Singh 2001; Yazdani 2004a; Yazdani 2006b). Recombinant PvDBPII elicits high-titer antibodies in small animals as well as nonhuman primates that block erythrocyte binding and invasion (Singh 2001; Yazdani 2004b; Grimberg 2007; Wiley 2011). A number of polymorphisms have been reported in PvDBPII in *P. vivax* field isolates from different geographical regions (Tsuboi 1994; Ampudia 1996; Xainli 2000; Cole-Tobian and King 2003; Ju 2013). However, determination of the three-dimensional structure of PvDBPII and mapping of the binding residues by site-directed mutagenesis indicates that key binding residues that make contact with Duffy antigen receptor are conserved in field isolates (van Buskirk 2004; Hans 2005; Singh 2006b).

An alternative model suggests that PvDBP forms a dimer and identifies additional binding residues for Duffy antigen (Batchelor 2011). Determination of the structure of PvDBPII in complex with its receptor is needed for a definitive understanding of the molecular interactions involved in this interaction. However, antibodies raised against PvDBPII block receptor-binding by diverse PvDBPII variants (Wiley 2011), which is consistent with the observation that key binding residues within PvDBPII are likely to be conserved (van Buskirk 2004; Hans 2005; Singh 2006b). Moreover, an immunoepidemiology study conducted in children residing in a malaria-endemic region of Papua New Guinea demonstrated that development of binding inhibitory antibodies against PvDBPII in children is associated with protection against *P. vivax* infection (King 2008). Such naturally acquired antibodies against PvDBPII block binding of diverse PvDBPII variants, confirming the observation that the binding residues are likely to be conserved (King 2008). These observations support the inclusion of PvDBPII in a vaccine against *P. vivax* malaria.

Although the central dogma has been that *P. vivax* erythrocyte invasion is completely dependent on the interaction between PvDBP and Duffy blood group antigen, studies from different

malaria-endemic regions have reported *P. vivax* infections in Duffy-negative individuals (Cavasini 2007; Menard 2010; Mendes 2011; Ngassa Mbenda 2014), suggesting that the parasite may have evolved alternative invasion pathways mediated by redundant ligand–receptor interactions. Moreover, genome sequencing has revealed the presence of 10 members of the family of reticulocyte-binding proteins (PvRBPs), the first members of which (PvRBP1 and PvRBP2) were shown to specifically bind reticulocytes and were thought to be responsible for the preferential invasion of reticulocytes by *P. vivax* (Galinski 1992; Carlton 2008).

In addition, genome sequencing of *P. vivax* field isolates has demonstrated the presence of a DBL homologue, which could play a role in invasion by Duffy-independent pathways (Menard 2013). Thus, like *P. falciparum*, it appears that *P. vivax* has also evolved redundancy to circumvent its sole dependence on the Duffy interaction (Mercereau-Puijalon 2010). Further work is in progress to elucidate the functional receptor binding domains of the PvRBPs that could, either individually or in combination with the PvDBP, serve as potent targets of antibody-mediated blockade of reticulocyte invasion. The PvRBPs, along with their homologues in *P. falciparum* known as the *P. falciparum* reticulocyte binding–like homologous (PfRH) proteins, are collectively known as the reticulocyte binding–like (RBL) family.

P. falciparum uses multiple invasion pathways that are mediated by members of the DBL and RBL families. EBA-175 binds sialic acid/glycophorin A on erythrocytes as receptors (Camus and Hadley 1985; Sim 1994). This invasion pathway is commonly used by diverse *P. falciparum* field isolates (Jiang 2011). The receptor-binding domain of EBA175 has been mapped to region II (EBA175-RII), which is composed of tandem DBL domains, F1 and F2 (Sim 1994). Antibodies raised against EBA175-RII or EBA175-F2 have been shown to block receptor-binding and invasion by diverse *P. falciparum* field isolates (Pandey 2002; Mamillapalli 2006; Jiang 2011).

Recombinant EBA175-RII formulated with alhydrogel was found to be immunogenic in a Phase I human trial and elicited invasion-inhibitory antibodies (El Sahly 2010). The *P. falciparum* vaccine candidate JAIVAC-1, which is a mixture of recombinant EBA175-F2 and recombinant $PfMSP1_{19}$ formulated with Montanide ISA720, has been tested in a Phase I human trial in healthy adults (Chitnis 2015). JAIVAC-1 was found to be safe and elicited antibodies against EBA175-F2 that recognized native EBA175 by IFA and blocked erythrocyte invasion by *P. falciparum* strains *in vitro* (Chitnis 2015). However, $PfMSP1_{19}$ was found to be poorly immunogenic and did not elicit antibodies that recognized the native antigen in *P. falciparum* schizonts or merozoites (Chitnis 2015).

It has been reported that antibodies against region III–V of EBA-175 that represent the non–receptor-binding domain of the parasite protein exhibit potent invasion-inhibitory activity (Lopaticki 2011; Healer 2013). This novel event suggests that regions other than the receptor-binding domains of these parasite adhesins could also be efficacious targets of antibody-mediated blockade of invasion and should be considered for evaluation as potential vaccine candidates. These studies support the inclusion of EBA175 in a blood-stage vaccine for *P. falciparum* malaria and indicate the need to find ways to make $PfMSP1_{19}$ more immunogenic, which is discussed later.

The *P. falciparum* reticulocyte-binding–like homologous (PfRH) family of erythrocyte-binding adhesins have attracted immense attention in the past decade as they have been identified as key determinants of different alternate invasion pathways. The PfRH family comprises five functional erythrocyte-binding proteins, PfRH1, PfRH2a, PfRH2b PfRH4, and PfRH5 (Gaur 2004). PfRH1 binds erythrocytes in a sialic acid–dependent manner (Gao 2008), whereas PfRH2 (Sahar 2011; Gunalan 2011), PfRH4 (Gaur 2007; Tham 2010), and PfRH5 (Hayton 2008; Baum 2009; Crosnier 2011; Reddy 2014) exhibit sialic acid–independent erythrocyte-binding activity. The sialic acid–independent receptors of PfRH4 and PfRH5 have been identified as complement receptor 1 (CR1) (Tham 2010) and basigin (CD147) (Crosnier 2011), respectively.

All PfRH proteins, with the exception of PfRH5 (63 kDa), are high-molecular-mass proteins on the order of 250 to 375 kDa (Gaur 2004; Gaur and Chitnis 2011). The receptor-binding domains of all PfRH proteins have been mapped, and antibodies against these respective functional domains exhibit both binding and invasion-inhibitory activity (Gaur 2004, 2007; Gao 2008; Sahar 2011; Triglia 2011; Reddy 2014). Several other novel parasite proteins have also been identified, such as apical asparagine-rich protein (PfAARP) (Wickramarachchi 2009) and thrombospondin-related adhesive merozoite proteins (PfTRAMP) (Siddiqui 2013), which are localized in the rhoptries (Gaur and Chitnis 2011) and of which AARP antibodies exhibit invasion inhibition (Wickramarachchi 2009; Pandey 2013; Reddy 2014).

Parasite ligands appear to be secreted sequentially from micronemes and rhoptries during invasion (Gaur and Chitnis, 2011; Sharma and Chitnis, 2013). Parasite ligands localized in micronemes and rhoptries are thus likely to mediate distinct sequential steps in invasion. It has also been reported through a number of studies that the EBA and PfRH family of proteins display a functional cooperativity (Stubbs 2005; Gaur 2006; Lopaticki 2011), which determines the phenotypic variations exhibited by different *P. falciparum* strains during erythrocyte invasion (Duraisingh 2003; Triglia 2005).

Previously, the classical essential blood-stage targets such as MSP-1 and AMA-1 had failed to elicit cross-strain–transcending invasion-inhibitory antibodies, primarily due to a high level of antigenic polymorphisms (Takala 2007, 2009; Duan 2008). The EBA and PfRH proteins show limited polymorphisms primarily due to their localization in the apical organelles (Rayner 2005; Jiang 2010). They translocate to the merozoite surface only during the later stages of erythrocyte invasion. Thus, with the exception of PfRH5, they are not essential, and antibodies targeting them individually do not block erythrocyte invasion efficiently (Gaur and Chitnis 2011). However, targeting a combination of such parasite ligands with antibodies synergistically inhibits erythrocyte invasion with high efficiency (Lopaticki 2011; Pandey 2013).

Antibody combinations against different microneme and rhoptry proteins such as EBA175 (PfF2 or RIII-V), PfRH1, PfRH2, PfRH4, and PfAARP were identified through a large screening exercise to potently inhibit *P. falciparum* growth *in vitro* (Lopaticki 2011; Pandey 2013). Importantly, these antibody combinations blocked *in vitro* growth of diverse *P. falciparum* strains, indicating that polymorphisms would not be a problem if these antigens are targeted in combination.

More recently, the PfRH5–basigin interaction was demonstrated to be essential in multiple *P. falciparum* strains (Crosnier 2011), and interestingly, individual antibodies against full-length PfRH5 generated through the adenoviral vector platform produced highly potent inhibition of erythrocyte invasion in a strain-transcending manner (Douglas 2011). PfRH5 thus emerged as the only adhesin among the EBA/PfRH families to elicit strain-transcending neutralizing antibodies. It was also reported that naturally acquired antibodies against PfRH5 were low in malaria-endemic regions of Kenya (Douglas 2011), with poor acquisition of anti-PfRH5 antibodies with age following repeated exposure to *P. falciparum* infection (Richards 2013). Although rare, acquisition of antibodies against PfRH5 is associated with protection against *P. falciparum* malaria, and anti–PfRH5-specific antibodies purified from human plasma exhibit potent invasion-inhibitory activity (Patel 2013). Antibodies raised against PfRH5 in laboratory animals using adenoviral vectors are more potent than AMA-1 antibodies in blocking erythrocyte invasion by multiple *P. falciparum* strains (Douglas 2011).

Following this study, a number of groups across the world have produced recombinant full-length PfRH5 either in *Escherichia coli* (Reddy 2014) or mammalian cells (Bustamante 2013) or baculovirus-based insect cells (Patel 2013) that have also similarly elicited potent parasite-neutralizing antibodies. Moreover, immunization of *Aotus* monkeys with recombinant full-length PfRH5 formulated with Freund's adjuvant elicited complete protection on challenge (Douglas 2015). In the same study, PfRH5 delivered through the chimpanzee adenoviral vector followed by a boost with MVA also protected the monkeys after challenge with a lower efficacy compared to the

formulation with Freund's adjuvant. These studies highlight PfRH5 as an attractive blood-stage vaccine target.

The inhibitory potential of PfRH5 antibodies is synergistically enhanced when combined with antibodies against other key merozoite antigens such as PfRH1, PfRH2, PfRH4, PfAARP, and PfEBA-175 (Williams 2012; Reddy 2014). PfRH5 exists on the merozoite surface as part of a large multiprotein complex comprising Ripr (RH5 interacting protein) (Chen 2011) and a novel GPI-linked protein known previously as CyRPA (cysteine-rich protective antigen) (Dreyer 2012), which is now functionally characterized as the RH5–Ripr membrane anchoring protein (RRMAP) (Reddy 2015). Antibodies against both Ripr (Chiu 2014) and CyRPA block erythrocyte invasion, with those against CyRPA exhibiting a potency similar to that reported with PfRH5 antibodies (Reddy 2015). Antibody combinations against PfRH5 and CyRPA also exhibit synergy in their invasion-inhibition potential against multiple *P. falciparum* strains (Reddy 2015). The three-dimensional crystal structure of PfRH5 has been reported (Wright 2014). Similar structure analysis of PfRipr, CyRPA, and the multiprotein complex would augment the design and development of potent blood-stage vaccines targeted at destabilizing the formation of the essential PfRH5 adhesion complex.

Although the ability to inhibit *P. falciparum* multiplication *in vitro* in growth-inhibition assays (GIA) is used as a criterion for down-selection of blood-stage vaccine candidates, it is not clear if GIA inhibition titers are surrogate markers for protection against *P. falciparum*. Although several molecular epidemiological studies have shown that the human plasma samples from people protected from the disease did exhibit GIA activity, it definitely did not appear to be sufficient for protection, because many plasma samples were found to exhibit GIA activity but the individuals were not protected against malaria (Duncan 2012). It is thus important to explore alternative bioassays for evaluation of antibodies raised against blood-stage antigens.

Genetic approaches have been used to produce *P. berghei* parasites that express chimeric MSP1 with $PbMSP1_{19}$ replaced with $PfMSP1_{19}$ (de Koning-Ward 2003). Such transgenic parasites can be used to test protection *in vivo* in mouse models. Following immunization of mice with $PfMSP1_{19}$- or $PfMSP1_{42}$-based constructs, mice can be challenged with transgenic *P. berghei* expressing chimeric MSP1 to evaluate *in vivo* efficacy. In addition to direct inhibition of invasion, such *in vivo* assays may allow evaluation of the role of alternative immune mechanisms that depend on presence of Fc receptor–positive immune cells for parasite clearance or growth inhibition (Bouharoun-Tayoun 1995; Pleass 2003). There have also been concentrated efforts to develop humanized mouse models that carry human erythrocytes and thus allow the propagation of *P. falciparum* parasites (Dreyer 2011; Vaughan 2012). Such models have also now been developed for the liver stage and should permit the *in vivo* evaluation of malaria vaccine candidates (Vaughan 2012).

In addition to directly blocking erythrocyte invasion with antibodies that target functional invasion-related proteins as described above, it is possible for antibodies that bind proteins on the merozoite surface to inhibit blood-stage parasite growth by antibody-dependent cellular mechanisms (ADCI) (Bouharoun-Tayoun 1995). ADCI requires presence of monocytes, and clearance is mediated by a soluble factor whose secretion is triggered by interaction of FcγRII receptors on monocytes with antibodies bound to merozoite antigens (Bouharoun-Tayoun 1995). *P. falciparum* merozoite antigens that serve as targets for ADCI as determined by *in vitro* parasite growth inhibition assays include PfMSP2, PfMSP3, and PfGLURP (McCarthy 2011; Oeuvray 1994; Singh 2009; Stubbs 2011; Theisen 1998). ADCI has been shown to be a potential protective mechanism in immune individuals residing in malaria-endemic regions (Druilhe and Perignon 1994). A humanized SCID mouse model has been developed to evaluate *P. falciparum* growth inhibition *in vivo* in presence and absence of monocytes (Badell 2000).

MSP2 is a 30-kDa GPI-anchored merozoite surface antigen (Sanders 2006; Yazdani 2006). MSP-2 antibodies have been observed to be strongly associated with protection against malaria (Metzger

2003) and display ADCI activity (Flueck 2009). Bacterially expressed MSP-2 was evaluated for its vaccine potential as a component of a combination B vaccine that comprised MSP-1, MSP-2, and a portion of RESA, which were formulated with Montanide ISA720 as the adjuvant (Genton 2002). Combination B was administered to Papua New Guinean children in a Phase IIb clinical trial that showed significant reductions in parasite densities (Genton 2002).

Merozoite surface protein 3 (MSP-3), a 48-kDa protein, is associated with the membrane of the free blood-stage malaria parasite (Oeuvray 1994). It is the first malaria vaccine candidate identified on the basis of the observation that it is responsible for the induction of cytophilic antibodies that mediate antibody-dependent monocyte-mediated growth inhibition of the parasite (Oeuvray 1994). Anti-MSP-3 antibodies, either naturally or elicited by immunization, killed parasites in the presence of monocytes (Oeuvray 1994; Bouharoun-Tayoun 1995). All recombinant protein and peptide formulations of MSP-3 were found to be immunogenic. In particular, a 70–amino acid–residues C- erminal region of MSP-3, which is highly conserved in various falciparum isolates was identified as a target for protective antibodies in humans (Singh 2004).

Immunization of human volunteers with a long synthetic peptide (LSP) derived from conserved regions of PfMSP3 (PfMSP3-LSP; amino acids 181–276) formulated with Montanide ISA720 or alhydrogel elicits antibodies that block *P. falciparum* blood-stage parasite growth in the presence of monocytes both *in vitro* and *in vivo* in the humanized SCID mouse model infected with *P. falciparum* (Audran 2005). Healthy, semi-immune adult male volunteers from a Phase I clinical trial with PfMSP3-LSP formulated with alhydrogel, who resided in an area with high malaria transmission in Burkina Faso (West Africa), were followed up to test for protection against *P. falciparum* malaria (Sirima 2007). Groups receiving the vaccine had lower incidence of *P. falciparum* malaria, suggesting that the PfMSP3-LSP can elicit protective immune responses (Sirima 2007; Nebie 2009).

In a double-blind, randomized, controlled-dose escalation Phase 1b trial in children aged 12 to 24 months in Tanzania, immunization with MSP-3-LSP adsorbed on aluminum hydroxide, indicated that the vaccine was safe and immunogenic (Lusingu 2009). A similar study in children of the same age group in Burkina Faso showed essentially the same results (Sirima 2009). In both these studies MSP-3 LPS elicited high levels of anti-MSP-3–specific IgG3 Abs, the isotype involved in ADCI-based killing of the parasite. A follow-up of the study in Burkina Faso showed that the incidence rates of clinical malaria were substantially lower in the vaccinated children than in the control group that received the hepatitis B vaccine (Sirima 2011).

The *P. falciparum* glutamate-rich protein (GLURP) is another antigen targeted by human antibodies that can kill the parasite by ADCI mechanisms. A recombinant hybrid protein comprising $GLURP_{25\text{-}500}$ and $MSP\text{-}3_{212\text{-}382}$ (GMZ2) was expressed in *Lactococcus lactis* and was found to be immunogenic in mice. Safety and immunogenicity studies of GMZ2 adjuvanted with aluminum hydroxide has shown it to be well tolerated and immunogenic in malaria-naïve adults from Germany as well as in malaria-exposed adults from Gabon (Esen 2009; Mordmuller 2010; Jepsen 2013). In a clinical phase 1b trial, GMZ2, adjuvanted in aluminum hydroxide, was found to immunogenic, well tolerated, and safe in 1- to 5-year-old malaria-exposed Gabonese children. Memory B-cells against GMZ2 were also reported to have developed in these GMZ2-vaccinated groups (Belard 2011). These trials point to the potential of MSP-3 and GLURP-like antigens that induce protective immune responses that function through ADCI mechanism. A combination of antigens that produce both cytophilic antibodies as well as invasion-inhibitory antibodies may be an attractive alternative for a blood-stage malaria vaccine.

Another novel approach for the development of new-generation blood-stage malaria vaccines involves the production of chimeric fusion proteins comprising more than one vaccine target antigen. These fusion proteins are expected to elicit immune responses to the different components that make up these chimeric vaccine target antigens. A fusion protein comprising apical membrane

antigen (PfAMA-1) (Faber 2007) fused to $PfMSP1_{19}$ significantly enhanced induction of antibody responses. Immunization with a chimeric *P. falciparum* vaccine containing $PfMSP1_{19}$ fused to PfMSP8 (rPfMSP1/8) and formulated with different adjuvants produced high titers of $PfMSP1_{19}$-specific antibodies. Formulations of PfMSP1-42 combined with PfMSP8 elicited antibodies that potently inhibited the *in vitro* growth of different strains of *P. falciparum* parasites.

Similarly, a fusion protein of *P. falciparum* merozoite surface protein-3 and glutamate-rich protein, namely, GMZ2, has been tested in humans and was found to be immunogenic, well tolerated, and safe in young children. A fusion protein containing a fragment of PfMSP3 with B- and T-cell epitopes fused to the C-terminal fragment of MSP1 ($PfMSP1_{19}$) has been developed. Immunization with this fusion protein (PfMSP-Fu24) elicits strong antibody responses to both components that exhibit significant growth-inhibitory activity that is mediated both by directly blocking its invasion of erythrocytes and by the ADCI mechanism (Gupta 2014). Of particular significance was the finding that antibody responses to $PfMSP1_{19}$ fragment were significantly higher than immunization with a physical mixture of the two components.

$PfMSP1_{19}$ is a highly conserved target of potent invasion inhibition. However, $PfMSP1_{19}$ is poorly immunogenic, as it lacks T-helper (T_h) epitopes. The MSP-3-$MSP\text{-}1_{19}$ fusion protein (PfMSPFu24) has circumvented this problem by inducing strong antibody responses with the help of T_h epitopes from PfMSP-3 (Mazumdar 2010; Gupta 2014). Antibodies against PfMSP-Fu24 elicit potent invasion-inhibitory antibodies (Mazumdar 2010; Gupta 2014). This chimeric protein represents the first example (Gupta 2014) of elicitation of antibody response of different functionality and is being taken forward for clinical development at ICGEB.

The pathology of severe malaria is attributed in significant measure to the ability of *P. falciparum* trophozoites and schizonts to bind vascular endothelium and sequester in the vasculature of diverse host organs. Adhesion to host endothelial receptors is mediated by the PfEMP1 family of variant surface antigens that are encoded by *var* genes (Miller 1994). Acquisition of antibodies against polymorphic PfEMP1 family members following infection with diverse *P. falciparum* strains over time is associated with protection against *P. falciparum* malaria (Miller 1994).

Such antibodies are primarily variant specific, and immune protection depends on acquisition of a repertoire of antibodies that recognize diverse PfEMP1 variant sequences (Bull 1998). However, in case of malaria during pregnancy, *P. falciparum*-infected trophozoites and schizonts primarily sequester in the placenta by adhesion to chondroitin sulfate (CSA) (Fried and Duffy 1996). Binding to CSA is mediated by *var* genes that are referred to as *var2csa* (Salanti 2004). Moreover, mothers who are exposed to placental malaria during pregnancy develop antibodies that react with heterologous CSA-binding *P. falciparum* late stages (Fried 1998; Barfod 2010). As a result, incidence of pathological outcomes of placental malaria, such as low birth weight and premature deliveries, are more common in primigravidas compared to secondigravidas and multigravidas. It should thus be possible to raise cross-reactive antibodies against *var2sa* that recognize diverse CSA-binding *P. falciparum* strains. Efforts to produce either full-length *var2csa* or CSA-binding domains of *var2csa* for use in preclinical and clinical studies are under way.

Transmission-blocking vaccines

As the mosquito takes a blood meal during a mosquito bite, it picks up gametocytes as well as any antibodies that may be present in host peripheral blood. Immunization with sexual- or mosquito-stage parasite antigens can be used to raise antibodies against antigens from these stages. Such antibodies will be taken up by the mosquitoes during their blood meal and can block progress of malaria parasite life-cycle stages in the mosquito. A membrane feeding assay (MFA) in which the

development of oocysts is scored after *Anopheles* mosquitoes are allowed to feed on *P. falciparum* blood-stage cultures containing gametocytes in presence or absence of antibodies against sexual- or mosquito-stage antigens is used to evaluate the efficacy of antibodies in blocking transmission. Individuals residing in malaria-endemic areas develop antibodies against gametes that block malaria transmission. The MFA has been successfully used both to identify potential transmission-blocking vaccine (TBV) candidate antigens and to evaluate immunogenicity of transmission-blocking vaccine candidates (Miura 2013b; Saul 2007). Such vaccines could be critical components of efforts to eliminate malaria.

The leading candidates for TBVs include *P. falciparum* ookinete surface proteins, Pfs25 and Pfs28 (Saxena 2007), and gamete surface proteins, Pfs48/45 (van Dijk 2001) and Pfs230 (Williamson 2003). Ookinete proteins such as Pfs25 and Pfs28 are not polymorphic because they are not under any immune pressure. Gamete proteins such as Pfs230 and Pfs48/45 are more polymorphic but contain conserved cysteine-rich domains with a characteristic 6-cysteine pattern. Immunization of small animals with these candidate antigens produced as recombinant proteins elicits antibodies with transmission-blocking activity. Another approach to block transmission is to target the vector instead of the parasite by immunizing against components of the mosquito midgut or salivary gland. The leading example of such an approach is the demonstration that immunization with recombinant Apn1 from the mosquito midgut blocks transmission of both *P. falciparum* and *P. vivax* (Dinglasan 2007).

Transmission-blocking vaccine candidates have been tested in early-phase clinical trials for safety and immunogenicity. A Phase I clinical trial with recombinant Pfs25 and its *P. vivax* homologue, Pvs25, formulated with a strong adjuvant, Montanide ISA51, had to be stopped due to reactogenicity issues related to the adjuvant formulation (Wu 2008). The challenge for development of TBVs is to develop delivery platforms and/or adjuvant formulations that are able to elicit high-titer antibodies in humans with significant transmission-blocking activity. Conjugation of Pfs25 to a protein carrier such as outer membrane protein of *Neisseria meningitides* serogroup B greatly improved immunogenicity when tested in nonhuman primates as alum formulations (Wu 2006). Importantly, antibodies inhibited transmission blocking in the MFA and were long lasting (Wu 2006). Similarly, conjugation of Pfs25 with nontoxic exoprotein A of *Pseudomonas aeruginosa* also elicits high-titer, long-lasting transmission-blocking antibodies (Kubler-Kielb 2007; Shimp 2013). Development of such novel approaches to improve immunogenicity of TBVs to elicit long-lasting high-titer antibodies with transmission-blocking activity is key to the success of these vaccines.

Live attenuated vaccines for malaria

As mentioned earlier, there is clear evidence that bites of irradiated *P. falciparum*–infected mosquitoes consistently protect against subsequent challenge with bites of sporozoite-infected mosquitoes. This observation suggests that it may be possible to use irradiated sporozoites as an attenuated vaccine. An alternative way to produce attenuated parasites is to use molecular genetic methods to knock out essential genes required for completion of liver stage and release of merozoites (Mueller 2005a; Mueller 2005b). Immunization with such genetically manipulated *P. berghei* sporozoites that infect hepatocytes but do not develop blood-stage infections completely protects mice against challenge with wild-type *P. berghei* sporozoites.

A cGMP process for producing irradiated *P. falciparum* sporozoites under aseptic conditions by dissection of salivary glands following infection of *Anopheles* mosquitoes with *P. falciparum* blood-stage cultures containing gametocytes by membrane feeding has been developed (Hoffman 2010).

Methods to cryopreserve and revive purified and irradiated *P. falciparum* sporozoites for immunization have also been standardized. Intradermal immunization with such attenuated *P. falciparum* sporozoites failed to elicit protection against subsequent challenge with bites of mosquitoes infected with *P. falciparum* sporozoites (Epstein 2011), although intravenous delivery elicited complete protection at the highest doses tested (Epstein 2011; Seder 2013). The duration of protection and protection against heterologous *P. falciparum* isolates elicited by irradiated sporozoites remains to be tested.

Conclusion

Progress has been made toward the development of a malaria vaccine (Gaur and Chauhan 2013). A partially effective vaccine for *P. falciparum* malaria, RTS,S, will complete Phase III clinical trials shortly and may begin the process of licensure. However, there is still a need to develop a vaccine with significantly better efficacy. It appears that a vaccine based on a single antigen that targets a single stage of the parasite life cycle is unlikely to provide high efficacy. It may be necessary to target multiple antigens and achieve synergy to provide high efficacy. *Plasmodium* antigens appear to be poorly immunogenic when formulated with commonly used human-compatible adjuvants. Breakthroughs are needed in delivery technologies and adjuvants to achieve robust immune responses against recombinant malaria vaccine candidate formulations. Finally, as the agenda of malaria research moves from control to elimination and eradication, it is necessary to consider how malaria vaccines can contribute to these efforts. At the same time, it is important to continue to work on development of vaccines that provide protection against malaria, as the burden of disease remains high despite recent gains.

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CHAPTER 20

Plasmodium vivax: Insights on burden and pathobiology

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Plasmodium vivax is increasingly recognized by the scientific community as a significant contributor to the global burden of malaria and as a potential cause of severe disease in humans. Its widespread geographical distribution and its capacity to relapse from its hypnozoite liver stages jeopardize control strategies and put it in the forefront, beside *Plasmodium falciparum*, of the human malaria parasites. In this chapter we review current knowledge regarding its estimated burden, its potential to cause disease (with particular emphasis on current knowledge regarding its most severe forms), the eventual relation of severity with chloroquine resistance, cytoadherence, and its pathophysiology. In this era of renewed and increasing research efforts in the malaria field, understanding how to adequately tackle this particular relapsing species has become fundamental if we want to achieve the ultimate goal of malaria eradication.

Burden of *Plasmodium vivax* infection and disease

Among the five *Plasmodium* species known to cause human disease, *Plasmodium falciparum* has justifiably attracted most attention, because it is directly responsible for the greatest proportion of severe morbidity and mortality. However, in recent years, the significant burden of *Plasmodium vivax*, the second most common cause of malaria, has been reappraised (Mendis 2001; Baird 2007; Price 2007b; Mueller 2009a). Indeed, this ubiquitous species is a threat to almost 40% of the world's population (Guerra 2010) and causes a minimum of 80 million clinical episodes annually (Mendis 2001; Price 2007b; Baird 2007). Its former weak contribution to the malaria burden in Africa, dictated by the generalized absence in most African populations of the Duffy trait (believed to be essential for the parasite's invasion of the host red cells), was compensated for by its impact in the rest of the world, particularly in the Asia-Pacific and the Americas, where it usually accounts for at least half of the malaria burden (Olivera-Ferreira 2010; World Malaria Report 2012).

When the famous German bacteriologist Robert Koch visited Java and New Guinea as part of his 1899–1900 malaria expedition, he conducted extensive surveys in indigenous populations and noted, "There are in malaria besides the pronounced cases, which are easily recognized by the clinical symptoms, very many which are not at all or at least not certainly recognisable, and can be diagnosed as genuine cases of malaria only by the discovery of malaria parasites in the

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blood." And, "If one confined one's attention to those patients who go of their own accord to the doctor, one would remove only a fraction of the malaria parasites" (Koch 1900). The most common *Plasmodium* species in his and subsequent New Guinea surveys by Dempwolff (Dempwolff 1904) was *P. vivax*.

In the time since Koch's initial observation, the importance of endemic asymptomatic *P. vivax* infections has been increasingly recognized. In large cross-sectional surveys in Papua New Guinea (PNG) populations with high prevalence of endemic infection, 16.6% to 18.3% of participants had microscopically patent *P. vivax* infections, with prevalence highest in children younger than 5 years (Genton 1995; Kasehagen 2006; Mueller 2009b; Arnott 2013). Of these infections, only 4% to 10% were symptomatic, compared to 6% to 12% in participants without *Plasmodium* infections (Mueller, unpublished data). The fraction of symptomatic infection increases to 25% among people living in the low-endemic highlands areas (Betuela 2012a). Similarly, high percentages of asymptomatic, microscopically patent *P. vivax* infection (range, 70%–97%) were also observed in Indonesian Papua, Solomon Islands, Vanuatu, and Brazil (Karnaya 2008; Harris 2010; Kinzer 2010; Alves 2002). With the development of polymerase chain reaction (PCR)-based malaria diagnosis (Snounou 1993), an even higher burden of low-density asymptomatic *P. vivax* infections became apparent. In PNG studies, prevalence by PCR was found to range from 27.1% to 35.6%, with age of peak prevalence shifting to children 5 to 10 years of age (Kasehagen 2006; Mueller 2009b; Arnott 2013). Similar increases in prevalence were also observed in other parts of the world (Snounou 1993; Alves 2002; Harris 2010; Kinzer 2010; Steenkeste 2010). Almost all of the PCR-positive, microscopically subpatent infections were not associated with clinical symptoms.

All these observations indicate that asymptomatic and subpatent infections constitute a major component of the global burden of *P. vivax* infections. Because even subpatent *P. vivax* infections may be efficiently transmitted (Jeffery 1952; Alves 2005; Bharti 2006; Bousema and Drakeley 2011), these infections are likely an important source of *P. vivax* transmission.

The rapid acquisition of clinical immunity to *P. vivax* is likely to be a major contributor to the high prevalence of asymptomatic infections in *P. vivax*–endemic areas. In highly endemic New Guinea, for instance, *P. vivax* is the predominant source of malaria infections and disease in children younger than 2 years (Senn 2012). From the second year of life, the incidence of *P. vivax* malaria starts to decrease rapidly, whereas *P. falciparum* incidence continues to increase until the fourth year (Lin 2010a) (Figure 20.1A). By age 5 to 14 years, children have acquired an almost complete immunity to clinical *P. vivax* but yet remain at considerable risk for *P. falciparum* illness despite a similar burden of *P. vivax* blood-stage infections (Figure 20.1B) (Michon 2007). Although *P. vivax* is a very significant source of severe illness in infants (Poespoprodjo 2009), the proportion of *P. vivax* infection manifesting with severe symptoms decreases rapidly with age (Tjitra 2008; Genton 2008), and in children older than1 year, the incidence of severe *P. vivax* illness is significantly lower than that due to *P. falciparum* (Manning 2012).

Interestingly, in children younger than 5 years the incidence of newly acquired, genetically distinct *P. vivax* and *P. falciparum* blood-stage infections (i.e., the molecular force-of-blood-stage infection [$_{mol}$FOB]) was strongly associated with increased risk of clinical malaria. *P. vivax* $_{mol}$FOB was, however, substantially higher than that of *P. falciparum* (Michon 2007; Mueller 2012; Koepfli 2013) despite comparable or higher entomological inoculation (Benet 2004; Hii 2001) rates for *P. falciparum*, whereas *P. falciparum* $_{mol}$FOB increased with increasing age in children aged 1 to 4 years (Mueller 2012) and remained constant thereafter. *P. vivax* $_{mol}$FOB did not change with increasing age (Michon 2007; Koepfli 2013).

The main reason, although yet to be formally proved, for the much higher $_{mol}$FOB is the ability of *P. vivax* to relapse from long-lasting, dormant liver-stages (i.e., hypnozoites). Following

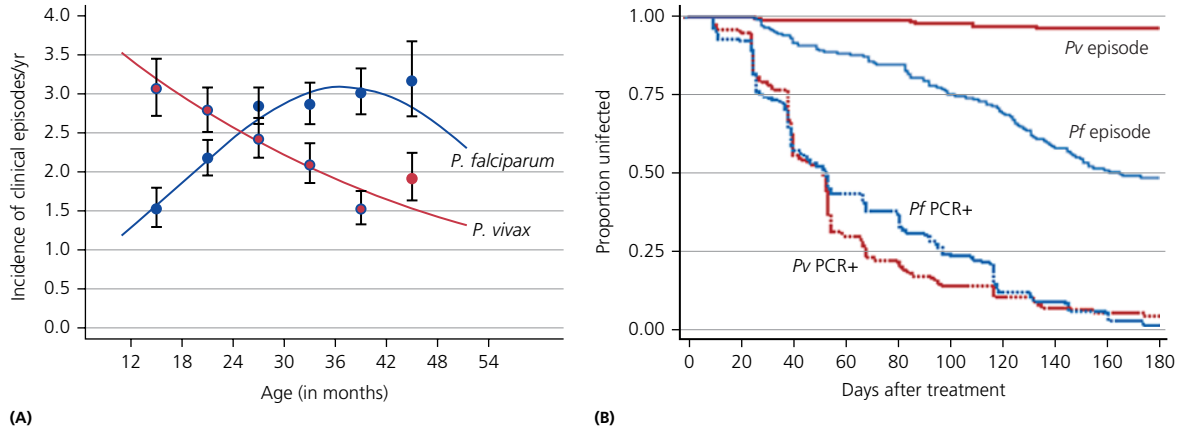

Figure 20.1 Evidence for rapid acquisition of immunity to *P. vivax* in PNG children. *A*, Incidence of malaria in a cohort of children 1 to 4 years (Lin 2010a). *B*, Time to first PCR-positive infection and clinical episode in children 5 to 14 years (Michon 2007).

treatment with a blood-stage drug, PNG children very rapidly reacquire *P. vivax* infections, with up to 80% of children having another vivax infection within 6 weeks of treatment (Karunajeewa 2008). High rates of recurrent *P. vivax* following treatment with different antimalarial drugs have been observed in other locations (Boulos 1991; Hutagalung 2005; Karunajeewa 2008; Ratcliff 2007). Similarly, when asymptomatic children were randomized to receive the presumptive anti-hypnozoite therapy primaquine, the incidence of *P. vivax* infection and disease in the following 3 months was reduced by at least 50% (Betuela 2012b). Based on these observations, it has been estimated that relapses contribute up to 80% of the *P. vivax* force-of-blood-stage infections (Baird 2008). Because *P. vivax* relapses even in moderately endemic areas tend to be genetically distinct from the primary infections (Chen 2007; Imwong 2007, 2012), such relapses might not only contribute to high higher burden of infection but also increase the antigenic repertoire that a child is exposed to over time.

This difference in natural acquisition of immunity between *P. vivax* and *P. falciparum* is not unique to highly endemic areas of New Guinea but was equally observed in areas with substantially lower transmission levels. In longitudinal studies carried out on the western border of Thailand (Phimpraphi 2008; Lawpoolsri 2010), in Sri Lanka (Mendis 2001), and Vanuatu (Maitland 1996) the incidence of *P. vivax* malaria also decreased significantly earlier than that of *P. falciparum* malaria, whereas in a Brazilian cohort among Amazonian settlers the risk of *P. vivax* malaria started decreasing after 5 to 6 years of residence in the endemic area compared to 8 to 9 years for *P. falciparum* (DaSilva-Nunes 2008). Equally, in the 1930s it was well known that *P. vivax* was a disease that predominantly affected children, and *P. falciparum* was the most common species causing infection among adolescents and adults in areas like Greece (Balfour 1935) and Puerto Rico (Earle 1939).

The data reviewed above clearly show the importance of subpatent asymptomatic infections and relapses to the *P. vivax* burden. In recent years, however, severe disease and death have been reported in exclusive association with *P. vivax*. We thus focus the remaining of this chapter on reviewing what little is known about clinical and molecular aspects of severe vivax disease, in particular in relation to the concomitant appearance of chloroquine-resistant parasites and the recent evidence demonstrating cytoadherence of *P. vivax*–infected reticulocytes to endothelial receptors.

Severe *Plasmodium vivax* malaria

Anyone having suffered a *P. vivax* clinical episode would seriously challenge its evaluation as benign, although disease attributed to this parasite has traditionally been regarded as such. However, 2005, a myriad of case reports and longitudinal studies (Kochar 2005 and 2010b; Barcus 2007; Genton 2008; Tjitra 2008; Poespoprodjo 2009) have convincingly demonstrated that this species can manifest with a broad clinical spectrum (including most common manifestations typically observed in *P. falciparum* infections) and is often involved in severe or even fatal episodes. Clinical and epidemiological observations (Genton 2008; Tjitra 2008; Poespoprodjo 2009; Kochar 2010b) describe incidence and mortality rates of *P. vivax* severe disease similar to those for *P. falciparum* in hospitalized patients. Although the initial reports of *P. vivax*–associated severe disease came from the Indian subcontinent (Kochar 2005, 2009, 2010b), such geographical localization of the disease potentially implying the existence of *P. vivax* strains with variable virulence (Anstey 2009), many other regions of the world where *P. vivax* malaria is endemic, including the Brazilian Amazon [1,2,3], the New Guinea Island (Genton 2008; Tjitra 2008; Poespoprodjo 2009; Barcus 2007; Lampah 2011), or Sudan (Mahgoub 2012), have also reported severe and life-threatening vivax episodes. Thus, the attribution of severity to specific virulent strains still needs to be confirmed.

Diagnosis of severe disease attributed exclusively to *P. vivax* necessarily implies the confirmation of *P. vivax* parasitemia and the exclusion of *P. falciparum* coinfection and other conditions with similar symptomatology. Microscopy differentiation of both species is not straightforward to less-experienced personnel, and *P. falciparum* parasites can remain occult due to their sequestration in the deep microvasculature of different organs. For this reason, molecular (PCR-based) diagnosis is believed to be the only reliable method for excluding *P. falciparum* coinfection in addition to confirming *P. vivax* mono-infection. As outlined above, people living in malaria-endemic areas progressively acquire immunity against the disease, tolerating infections without developing symptoms. Incidental parasitemia (both patent and subpatent) consequently can distort the diagnostic certainty among symptomatic patients, as a result of other infections or conditions. Thus, without a careful exclusion of other concomitant comorbidities such as dengue (Magalhaes 2012), leptospirosis, viral hepatitis, or bacterial sepsis, among others, establishing the sole *P. vivax* malaria–attributable fraction of disease (Bejon 2007) is challenging. Although none of the longitudinal studies conducted in New Guinea were able to reliably confirm *P. vivax* mono-infection and convincingly exclude other comorbidities, seriously hindering their validity, recent studies in India (Kochar 2009), Brazil (Alexandre 2010) and PNG (Manning 2011) have done so and confirmed the relation of *P. vivax* with severe disease. Furthermore, complete postmortem characterization of 17 patients with clinical and parasitological diagnosis of *P. vivax* malaria has confirmed the extent to which this parasite species can kill (Lacerda 2012a).

In the preantibiotic era, when *P. vivax* was endemic in most of Europe, the relapsing fevers caused by the parasite were heavily debilitating and carried a chronic significant health burden. However, and in parallel with the improvement of socioeconomic conditions, the prognosis of *P. vivax*–related disease seemed to ameliorate. Malariotherapy studies, in which neuropsychiatric patients were deliberately infected with *P. vivax* so that the fevers caused would act as a treatment of their psychiatric condition, showed very little severe disease, and this seems generally also true for *P. vivax* transmitted in temperate areas or *P. vivax*–imported disease in travelers (Muhlberger 2004). *P. vivax* epidemics sometimes occur, but generally they do not carry the high mortality burden of their *P. falciparum* counterparts. Moreover, relapse patterns (conditioned by the reactivation of liver dormant stages) can differ substantially according to the geographic region and can be very sporadic or extremely frequent and persistent (White 2011). Put together, all these observations seem to support the exceptionality of severe disease in patients with an adequate baseline health status, without

concomitant chronic conditions, or not exposed repeatedly to malarial infections, relapses, or the heavy burden of micronutrient deficiencies or other infections. In contrast, chronic exposure coupled with a poorer baseline health status and the concomitant presence of high resistance to standard treatments (Tjitra 2008; Garg 2012), as occurs in some areas such as New Guinea, could ease the way toward more-severe outcomes.

Whether independently or synergistically with other conditions, it is now evident that *P. vivax* induces potentially lethal clinical episodes, with a wide variety of different clinical manifestations. These include acute respiratory illness such as pulmonary edema or acute respiratory distress syndrome (Lawn 2003; Lomar 2005; Price 2007a), neurological disturbances such as altered consciousness or repeated convulsions, and acute kidney failure (Kute 2012). Blood cell deficiencies are another class of manifestation. They include anemia (Rodriguez-Morales 2006; Douglas 2012; Manning 2012), thrombocytopenia (Aggarwal 2005; Harish and Gupta 2009; Lacerda 2011), and coagulation abnormalities (Sarkar 2012). Splenic dysfunctions (Imbert 2009; Lacerda 2012a), shock (Kumar 2007), and multiorgan failure are other manifestations. *P. vivax* infections during pregnancy (Lacerda 2012b) can cause pernicious effects on the fetus and the newborn, including congenital malaria (Pineros-Jimenez 2008), low fetal birth weight, and subsequent increased infant morbidity and mortality (Anstey 2012).

One of the most common complications seen among *P. vivax* patients is not a direct effect of the infection *per se*, but rather the consequence of an adverse event triggered by the drugs required to treat it. Indeed, life-threatening hemolysis (Lacerda 2012a) leading to profound and acute anemia can occur in glucose-6-phosphate-dehydrogenase (G6PD)-deficient patients who receive primaquine (Clayman 1952; Vale 2009). Primaquine is an 8-aminoquinoline drug necessary for the radical cure of dormant liver hypnozoites to prevent relapses, but this drug should be withheld until G6PD activity can be confirmed.

Considering all these different clinical manifestations, the definition of severe malaria, historically relying on the World Health Organization's (WHO) proposed case definitions, which originally were only envisaged for *P. falciparum*, also need to be revisited to include any particularities related to *P. vivax*. However, all the recent literature on complicated *P. vivax* cases refers to these criteria, allowing comparison among distinct epidemiological sites. At least in children, WHO criteria for severity were highly associated with hospitalization in the intensive care unit (ICU), a true surrogate of severity (Lanca 2012).

Understanding how the parasite causes severe disease challenges our current comprehension of the pathophysiological mechanisms involved in these processes (Anstey 2009), and there is a need to identify the differential impact of host, parasite, and external factors. *Plasmodium falciparum* causes clinical symptoms in relation to two very different although possibly synergistic mechanisms (Clark 1999): the activation of proinflammatory responses (with the corresponding cytokine release) and the significant obstruction of blood flow at the microvasculature level ensuing from cytoadherence and sequestration of parasitized red blood cells. In *P. vivax* infections, the pyrogenic threshold (parasitemia needed to cause fever) and the parasite biomass are significantly lower than in *P. falciparum* infections, but the overall inflammatory response triggered by the infection is of greater magnitude, and it seems to play a critical role in clinical symptomatology (Anstey 2012). Localized exacerbations of this acute inflammatory response, leading to the destruction of parasitized red blood cells in the lung microvasculature, have been proposed to cause acute respiratory distress syndrome (ARDS) in adults (Figure 20.2) after the beginning of antimalarial therapy in otherwise uncomplicated *P. vivax* cases (Price 2007a). In children, however, ARDS is a rare entity, but the *P. vivax* clinical presentation described in hospital-based longitudinal studies (Genton 2008; Tjitra 2008; Kochar 2010a) is often indistinguishable from that of *P. falciparum*, with a high incidence of other severe respiratory symptoms and often coexisting with severe effects on other organs, irrespective of the time of treatment.

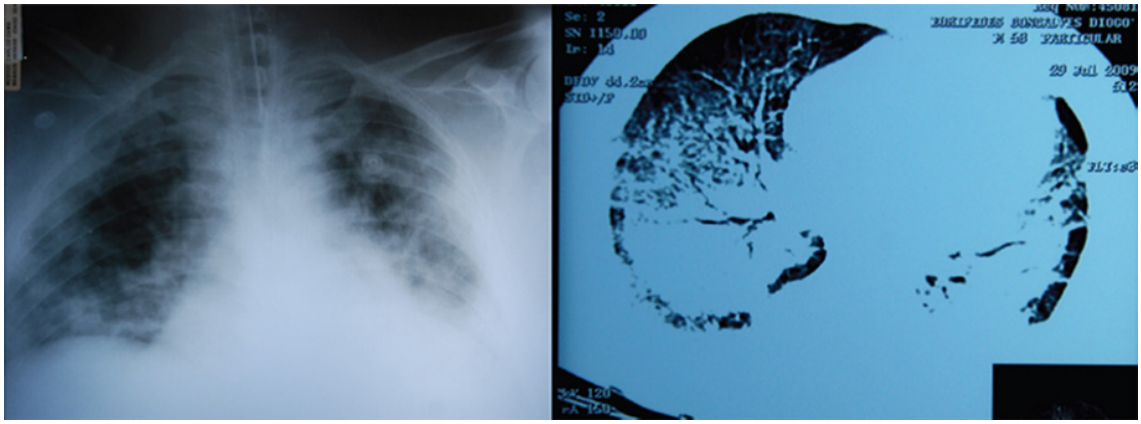

Figure 20.2 Thorax x-rays and computed tomography of a 40-year-old patient with diagnosis of *P. vivax* monoinfection by TBS and PCR, in which other chronic and acute comorbidities were ruled out, developing ARDS after chloroquine was started. Phenomena behind this common clinical complication, such as cytoahesion, still need to be clarified.

This suggests that cytoadherence and sequestration phenomena, critical in the pathophysiology of severe *P. falciparum* malaria but long-believed not to occur in *P. vivax* infections, might also play an important role, either *per se* or as a means of localizing a more-targeted inflammatory response to parasites adhering in certain organs. The recent confirmation of the occurrence of such phenomena in *P. vivax* infections (Carvalho 2010) (albeit with a lower frequency but with similar intensity), supported with further clinical observations (Anstey 2007), has opened a new pathway to explore the pathophysiology of disease caused by *P. vivax*.

Molecular basis of severe disease

The epidemiological and clinical data reviewed above demonstrated that natural infections of *P. vivax* can lead to severe clinical complications, including death, exclusively associated with this species. These complications involve a variety of clinical syndromes (Lacerda 2012b), and several reviews on different aspects of their pathophysiology have been published (Anstey 2009 and 2012; Price 2009; Douglas 2012). We focus here on two aspects of infection, chloroquine resistance and cytoadherence, which have been associated with severe *P. falciparum* malaria with devastating effects in public health and malaria mortality in Africa (Trape 2001; Miller 2002).

Chloroquine resistance

Chloroquine remained the first-line treatment against *P. falciparum* until resistance (CQR) to this unique, cheap, and safe antimalarial appeared independently in the late 1950s in Southeast Asia and South America and rapidly spread to other areas of Asia, Oceania, and Africa and throughout the world (Wellems and Plowe 2001). Noticeably, the appearance of CQR was concomitant with an increase in mortality, indicating a temporal association of CQR and severity of disease. This was first reported in a meticulous prospective longitudinal study in three different villages in Senegal, where malaria-specific mortality was recorded before, during, and after the appearance of CQR (Trape 1998). The results unambiguously demonstrated that CQR was temporally associated with a dramatic increase of malaria mortality particularly among children younger than 5 years.

Other confounders such as climate changes and intensity of transmission were excluded because, for instance, the village with the highest mortality rate had the lowest transmission. Afterward, a review of the literature on malaria in African countries where CQR had been registered further demonstrated the temporal association of CQR and malaria severity and mortality, forcing a change of public health policy to adopt antimalarial combination therapies (Trape 2001). This policy has been adopted in most *P. falciparum*–endemic regions (Lin 2010b; Mita and Tanabe 2012).

Similar to *P. falciparum* in Africa, the appearance of severe P. *vivax* malaria seemed concomitant with the spread of CQR. This was first reported in retrospective studies conducted in Papua, Indonesia, where pediatric cases of severe *P. vivax* malaria, including deaths, were observed to increase in children younger than 1 year in areas where high levels of CQR had been established (Tjitra 2008). The putative association of severe *P. vivax* malaria and CQR was further suggested by observations from Papua New Guinea, where CQR was originally reported and where severe disease decreased after combination therapy was introduced in the country (Schuurkamp 1992; Genton 2008). In addition, there are increasing reports on severe vivax disease exclusively associated with *P. vivax* from other endemic areas where CQR has started to appear (Price 2009). All together, these data strongly indicate that, similar to severe *P. falciparum* disease, severe *P. vivax* disease is likely associated with CQR; however, prospective longitudinal studies in areas where CQR is currently being spread are needed to statistically confirm this association. Data from India, on the other hand, clearly justify further investigation of this association, because India was one of the first countries to report vivax severity and CQR has not been found so far. It is possible that these are merely ecological associations, without any causality, or may even refer to a publication bias in areas devoted to more research.

Monitoring the spread of CQR in *P. vivax* is essential for surveillance and to facilitate studies on determining whether there is direct association of CQR and severe vivax disease. Mutations and copy-number variation of *P. falciparum* genes associated with CQR, mainly the chloroquine-resistance transporter gene, *pfcrt*, and the multidrug resistance gene, *pfmdr1*, have been used for these studies and are currently used as surrogate markers, albeit variably, of CQR (Valderramos and Fidock 2006). Studies on polymorphisms and copy number of the orthologous genes in *P. vivax*, *pvcrt-o* and *pvmdr1*, yield contrasting results compared to those obtained in *P. falciparum*. Thus, polymorphisms of *pvcrt-o* from different endemic regions of Southeast Asia and the Americas have all failed to associate any one particular mutation with resistance. Studies with *pvmdr1* have given contradictory results, because the Y976F mutation seems to be associated with CQR in some parts of Asia but not elsewhere (Barnadas 2008; Orjuela-Sanchez 2009; Lu 2011 and 2012) . For all other polymorphisms, however, there is no association with CQR. Gene amplification has not been observed in *pvcrt-o*, whereas amplifications of *pvmdr1* remain relatively rare and were associated with CQR only in one study (Suwanarusk 2008; Jovel 2011; Price 2012; Vatgas-Rodriguez 2012).

To understand whether expression levels rather than mutations of *pvcrt-o* and *pvmdr1* were associated with CQR, both genes were expressed in *trans* at different levels in *P. falciparum* transgenic lines of the chloroquine-sensitive 3D7 line (Sa 2006). Results from these experiments demonstrated that the transgenic line expressing the highest levels of *pvcrt-o* conferred a 2.2-fold increase in CQR and that such phenotype was reversible by verapamil. Noticeably, expression levels of *pvcrt-o* in parasites obtained from a first-time infected tourist who on return to Europe developed ARDS exclusively associated with *P. vivax* malaria were 21-fold higher than those obtained from three different patients with mild disease (Fernandez- Becerra 2009). Similar results have been observed in CQR patients from a cohort study in Brazil (Melo 2014). It is thus tempting to speculate that increased expression levels rather than mutations of *pvcrt-o* confer CQR and that such resistance is associated with disease severity. In the absence of other supporting data, this remains to be determined. Further studies to determine if *pvcrt-o* expression levels can be used as a molecular marker of CQR and/or severe vivax disease are necessary.

Besides gene polymorphisms, copy number, and expression levels, other non–mutually excluding explanations of the association of severe disease and CQR can be considered. Thus, although higher parasite biomass has not been directly shown to be associated with severe vivax disease, retrospective analyses of parasitemias in different studies reporting severity seem to indicate an increase in parasitemia (Baird 2013). In addition, *in vitro* studies have also shown that CQR parasites grow faster than chloroquine-susceptible parasites (Russell 2008). These explanations, however, remain questionable, because there are reports of severe vivax disease with no increase in parasitemia (Genton 2008). It is also likely that wild isolates of *P. vivax* have difference in virulence; indeed, studies with neurosyphilitic patients clearly showed that different strains were associated with differences in mortality (Snounou 2013). Similar studies from field-clinical isolates are currently unreported. These data strongly indicate that the appearance of severe *P. vivax* malaria was concomitant with the appearance of CQR parasites. Further studies in these emerging research areas are thus a priority to avoid a public health scenario similar to that of CQR and increased mortality in *P. falciparum*.

Cytoadherence

Severe disease in *P. falciparum* malaria is multifactorial, involving host and parasite factors (Miller 2002). Central to this pathology, however, are the phenomena of sequestration and of antigenic variation (Marchiafava 1894; Brown and Brown 1965). Sequestration is due to the capacity of infected red blood cells (iRBCs) containing mature stages to expose variant proteins serving as ligands for binding to endothelial receptors (Miller 1965, 2002). Cytoadherence in turn seems to obstruct blood microcirculation, causing local hypoxia and organ dysfunction. These conditions seem to be aggravated by further adherence of uninfected RBCs to iRBCs, a phenomenon known as *rosetting* (Handunnetti 1989; Udomsanpetch 1995), and by adherence of iRBCs to platelets, a phenomenon known as *platelet-mediated clumping* (Pain 2001; Rowe 2009). *Antigenic variation* refers to the capacity of the parasite to switch variant antigens at the surface of iRBCs, rendering immune responses insufficient to control infections (Guizetti and Scherf 2013; Borst 1995).

The search for genes encoding virulent determinants responsible for cytoadherence and antigenic variation first identified a subtelomeric multigene family, called the *var* gene family, encoding surface variant proteins collectively referred as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Baruch 1995; Smith 1995; Su 1995). There are close to 60 copies of *var* genes per haploid genome, and they present a common structure consisting of two exons: a small 5′-end exon encoding no canonical secretory signals and a second exon encoding several adhesion domains, such as the ICAM1, VCAM, and CSA, shown to act as ligands for human endothelial receptors (Smith 2001; Rowe 2009; Craig 2012). The landmark publication describing the complete genome sequence of the *P. falciparum* 3D7 strain demonstrated that in addition to *var* genes, other multigene families such as SURFIN, Pfmc-2TM, and STEVOR, were also located at subtelomeric ends (Gardner 2002). These other families have also been shown to contain adhesion domains and to be associated with pathology (Chen 2000). Thus, *P. falciparum* has clustered multigene families encoding virulent determinants responsible for phenomena associated with pathology at chromosome ends.

In the absence of a continuous *in vitro* culture system of blood stages from where to obtain unlimited parasite material, a chromosome end from a *P. vivax* isolate obtained from a single patient was cloned into a yeast artificial chromosome (Camargo 1997). Characterization of its primary structure revealed that, similar to *P. falciparum, P. vivax* contained a major subtelomeric variant multigene superfamily termed *vir* (*P. vivax* variant genes) (del Portillo 2001). Genes were composed of three exons, a 5′-end small exon with no canonical signal peptide sequences, a middle highly variant exon predicted to be exposed at the surface of infected reticulocytes, and a 3′-end exon containing a putative cytosolic domain. The completion of the genome sequence of the *P. vivax* Salvador I strain

allowed the annotation of the complete *vir* gene repertoire identifying 347 genes subdivided into different subfamilies termed A through H (Carlton 2008). Notably, the sequence also revealed the existence of other multigene families, termed the *P. vivax* fam families, whose function remains for the most unknown (Carlton 2008).

A new computational approach has redefined the *vir* gene superfamily and revisited the chromosome ends of malaria parasites (Lopez 2013). This algorithm robustly identified all previously described *P. falciparum* subtelomeric multigene families and demonstrated that *P. vivax* has evolved a more complex multigene-family structure at chromosomal ends (Figure 20.3). Thus, like *P. falciparum, P. vivax* has clustered multigene variant gene families at subtelomeric regions, suggesting a role in pathology through antigenic variation and cytoadherence.

Using isolates from *P. vivax* patients in Thailand and similar approaches as those used for studies of cytoadhesion in *P. falciparum*, White and coworkers failed to observe cytoadherence of *P. vivax*–infected reticulocytes to human umbilical vein endothelial cells, to C32 melanoma cells, to platelets, or to purified adhesion receptor molecule CD36 (Udomsanpetch 1995). These results, together with the presence of mature asexual blood stages in the peripheral blood of *P. vivax* patients, contributed to the conclusion that there is no sequestration in *P. vivax*. Yet, these data are difficult to reconcile with the evolution of so many different subtelomeric variant multigene families containing predicted adhesin domains.

To determine whether cytoadherence was exclusive to *P. falciparum*, studies revisited the adherence properties of *P. vivax*–infected reticulocytes from patients in Brazil to endothelial pulmonary cells, brain cells, and placenta (Carvalho 2010). These tissues were chosen because, respectively, ARDS, coma, and pregnancy-associated malaria are clinical syndromes often described in severe vivax disease. Unlike what had been observed in Thailand, *P. vivax*–infected reticulocytes from patients in Brazil cytoadhered to human tissue of these different origins under static conditions, albeit at lower levels than those observed for *P. falciparum*. Under flow physiological conditions, however, the strength of these interactions was the same for both species. These studies also demonstrated that *P. vivax*–infected reticulocytes were able to adhere to COS cells expressing the ICAM1 and CD36 receptors (Carvalho 2010), both known to be involved in adherence of *P. falciparum*. New *ex vivo* adhesion assays using *P. vivax* isolates from patients in Thailand demonstrated binding to placental cryosections, to CSA, and to hyaluronic acid, all known to be involved in adhesion of *P. falciparum* to the placenta (Chotivanich 2012). In contrast to the study from Brazil (Carvalho 2010), binding was not observed to immobilize CD36, ICAM1, or thrombospondin. The reasons for this discrepancy are currently unknown, but binding assays to ICAM1 and CD36 performed in Brazil were performed on COS cells expressing these receptors, whereas assays in Thailand were performed on immobilized purified receptors. Despite this discrepancy, these results strongly suggest that cytoadherence is not exclusive to *P. falciparum*, even though in the few studies in which tissue samples from patients with *P. vivax* infection are available, the phenomenon of cytoadherence is not as evident as that described in the 19th century by Marchiafava and Bignani for *P. falciparum*.

Adhesin domains from *P. falciparum* variant proteins, in particular those from PfEMP1 proteins, have been identified as ligands to different endothelial receptors (Smith 1995; Chen 2000). To identify ligands of *P. vivax*, cytoadherence inhibition assays of *P. vivax*-infected reticulocytes to COS cells expressing different receptors were performed using polyclonal monospecific IgG antibodies raised against two different VIR proteins (Carvalho 2010). It was reasoned that VIR proteins, due to their variant nature, subcellular localization, and genome organization, could mediate cytoadherence. Results showed that anti-VIR antibodies were able to specifically inhibit, albeit variably, this binding, suggesting that they contain adhesion domains (Carvalho 2010). More-direct and functional evidence was reported using a heterologous reverse genetics approach (Bernabeu 2011). Thus, VIR proteins with predicted differences in structure and subcellular locations were expressed in *trans* in

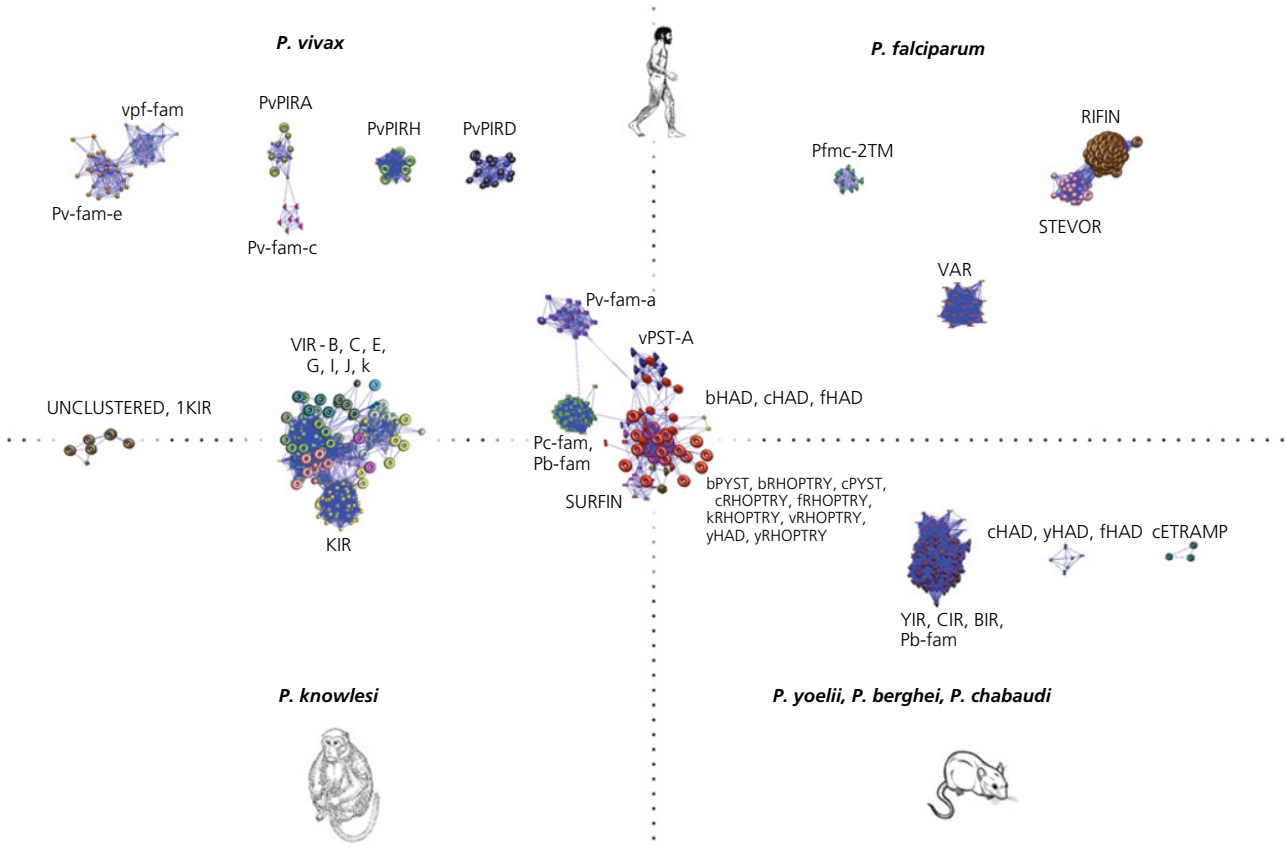

Figure 20.3 Subtelomeric multigene families of *Plasmodium vivax* and *P. falciparum*. Protein similarity relations among *P. vivax* and *P. falciparum* were obtained when running a new clustering procedure (Lopez 2013) over the subtelomeric families of *P. vivax* (Pv or v): VIR, PvPIRA-D-H, Pv-fam, v-Pf-fam, vRHOPTRY, v-PST-A, and *P. falciparum* (Pf or f): VAR, RIFIN, STEVOR, Pfmc-2TM, SURFIN, fRHOPTRY, fHAD (Carlton 2008). The position of the clusters with respect to the axes is just a qualitative representation; axes do not represent any metric. Graph figures were obtained using BioLayout (Crooks 2004).

P. falciparum. One of the transgenic lines exposed a VIR protein at the surface of iRBCs, allowing testing its binding capacity to different endothelial receptors. Under physiologically relevant flow conditions, such transgenic lines specifically adhere to COS cells expressing the ICAM1 receptor, which is already shown to be involved in pediatric severe *P. falciparum* malaria (Craig 2012). The binding was specifically inhibited by polyclonal antibodies raised against long synthetic peptides representing conserved motifs of VIR proteins (Bernabeu 2011). These data demonstrate that *P. vivax*–infected reticulocytes are able to cytoadhere to different endothelial receptors and that such adherence is at least partly and specifically mediated by VIR proteins.

Defined as the formation of rosettes due to adhesion of uninfected red blood cells with erythrocytes infected with mature forms of the parasite, rosetting is another cytoadherence mechanism known to be involved in disease severity in *P. falciparum* (David 1988; Udomsanpetch 1995). Rosetting seems to increase microvascular obstruction of the blood flow, and according to most, but not all, studies it is common in patients with severe or complicated malaria (Chen 2000; Rowe 2009). In addition, it is postulated that rosetting facilitates the rapid invasion of uninfected erythrocytes and protects iRBCs from destruction by macrophages, but this remains to be confirmed.

The capacity of *P. vivax*–infected reticulocytes to form rosettes was originally observed in Thailand, where two to twelve RBCs bound to each *P. vivax*–infected reticulocyte containing mature trophozoites or schizonts (Udomsanpetch 1995). Roughly 71% (25/35) of the isolates tested formed rosettes, suggesting that this was a common adhesive phenotype during infections. Rosetting was also reported from two independent studies using isolates from Thailand (Russell 2011; Chotivanich 2012). All 19 (100%) isolates from one study (Chotivanich 2012) and 30 of 33 (93%) from the other study (Russell 2011) claimed formation of rosettes, and our own unpublished observations confirm a large percentage of isolates from Brazil have rosetting capacity. These results clearly indicate that rosetting is the most common cytoadherence phenotype observed in natural infections with *P. vivax*; however, its role in pathology remains to be determined.

Platelet-mediated clumping is the third adhesion phenotype in malaria and refers to the capacity of iRBCs to cytoadhere to platelets forming mixed clumps *in vitro* (Pain 2001). Its role in pathology remains unclear, although several but not all reports demonstrate an association of platelet-mediated clumping with severe *P. falciparum* malaria (Rowe 2009). The role of platelets in cytoadhesion in *P. vivax* is unexplored. The peripheral number of platelets during the acute malarial episode seems to be similar between *P. vivax* and *P. falciparum* infection, but it is not known what the relationship is between thrombocytopenia and local inflammatory properties of these particles, including platelet-derived microparticles (Campos 2010).

Even though the evidence presented above strongly supports cytoadherence in *P. vivax*, the extent that such adherence phenotype is associated with pathology and more specifically with severe disease remains to be determined. Autopsy studies indicate overall little evidence of parasite accumulation in the inner organs, despite some evidence of sequestration in lungs from patients who died with negative peripheral parasitemia (Anstey 2012; Lacerda 2012a). A confounder of these studies is the concomitance of other infectious diseases contributing to death. It is important to recall here that *P. vivax*–infected reticulocytes are well known for their fragility upon manipulations and that antimalarial treatment as short as 3 hours removes adherent infected cells (Pongponratn 2003). In this sense, it is noteworthy that an autopsy of a patient dying of ARDS reported presence of scanty parasites in the lungs (Valecha 2009). This result is mainly used as an argument to claim that "sequestration mediated pathology in *P. vivax* malaria is at best modest" (Anstey 2012). Most relevant, in a well-conducted case-series study of autopsies from children dying of cerebral falciparum malaria, the main conclusion was that sequestration is commonly but not invariably associated with detectable intra- and perivascular pathology (Taylor 2004). Prospective autopsy studies from untreated and well-preserved organs in patients exclusively dying of *P. vivax* malaria are therefore needed to determine the exact role of sequestration in pathology of *P. vivax*.

In summary, the extent that cytoadherence, including rosetting and platelet-mediated clumping, contribute to severe vivax malaria remains to be determined. Yet, it is clear that these adhesive phenotypes are not exclusive to *P. falciparum*. Moreover, among *Plasmodium* species, *P. vivax* has evolved the largest number of subtelomeric variant gene families, and many of them encode predicted adhesins. Unveiling the role of these multigene families in infection is therefore a major key gap in knowledge of *P. vivax*. Technological advances in the development of a continuous *in vitro* culture system for blood stages and the possibility of applying reverse genetics in this species seem to indicate that tools will be soon available to accelerate answers to this fundamental question. In turn, it can lead to the identification of new antigens for use as vaccine candidates and the development of alternative public health control strategies.

Concluding remarks and outstanding research questions

These observations highlight the changing appreciation of both the burden of *P. vivax* and our rapidly expanding knowledge of *P. vivax* host–parasite interactions. Even though it causes a lower overall burden of severe disease than that ensuing from *P. falciparum* (Manning 2012), *P. vivax* can certainly no longer be considered benign, and both its clinical and economic burden are high (Price 2007b; Baird 2013). Equally important, it has become increasingly evident that in co-endemic areas where control has been intensified, *P. vivax* tends to be more resistant to control and remains the main obstacle to achieving eradication in many areas of the Asia-Pacific and Americas (Alonso 2011; World Malaria Report 2012). As a consequence, interest is greatly increasing in improving *P. vivax* treatment and control methods as well as in basic research into *P. vivax* host–parasite biology.

Of particular importance will be determining the role of cytoadherence in severe disease, the type of severity most associated to cytoadherence, and the extent to which severe disease is linked to CQR. Equally, gaining a more in-depth understanding of the real contribution of relapses to the burden of *P. vivax* and their impact in terms of acquisition of immunity is essential. Despite the long-standing absence of a stable, continuous *in vitro* culture system, it is possible to continue to deepen our understanding of these and other aspects of *P. vivax* burden and pathophysiology by combining *ex vivo* laboratory studies with well-designed population-based and clinical studies.

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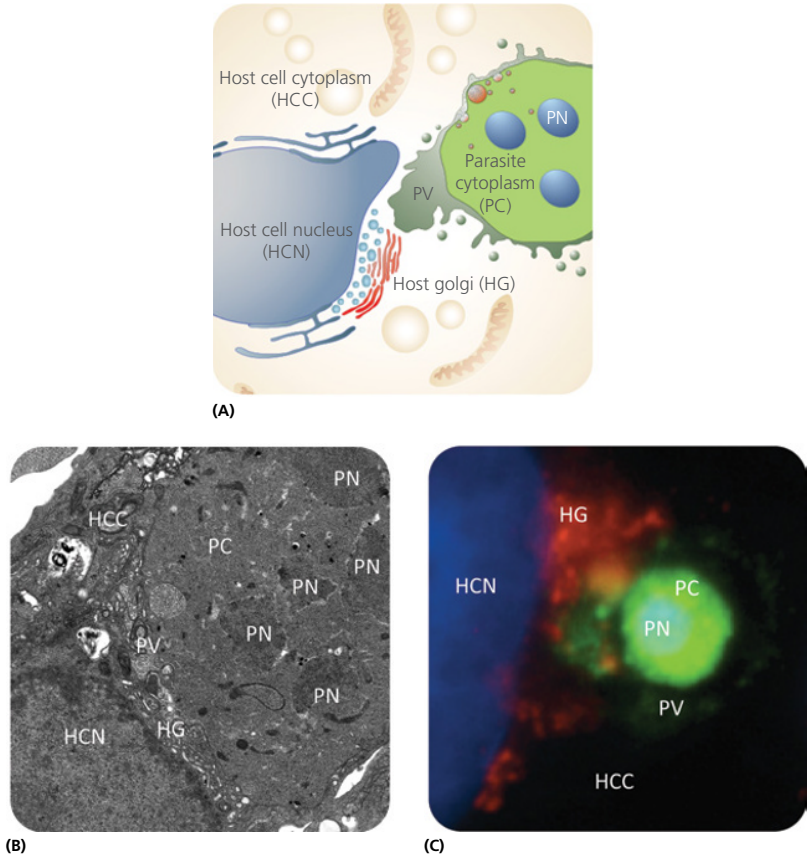

Figure 2.3 *P. berghei* schizont develops close to the host cell Golgi. A schematic representation *(A)*, a TEM image *(B)*, and an IFA image *(C)* are shown. HCC: host cell cytoplasm; HCN: host cell nucleus, HG: host Golgi; PC: parasite cytoplasm; PN: parasite nucleus; PV: parasitophorous vacuole. For the IFA, HepG2 cells were infected with *P. berghei* sporozoites and fixed 16 hpi. Staining of the host-cell Golgi was with an anti-BIP antibody *(red)* and the parasite was stained with an anti-PbICP antiserum *(green)*. DNA was visualized with DAPI.

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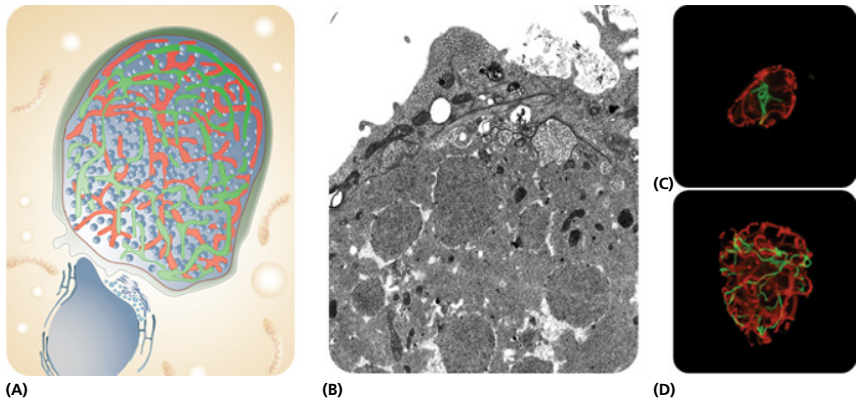

Figure 2.4 Organelle development in *P. berghei* liver schizonts. *A,* Scheme of a schizont with many nuclei *(blue),* one mitochondrion *(red),* and one apicoplast *(green). B,* TEM image showing a cross section of a schizont with mitochondria and apicoplast. *C* and *D,* Live imaging of early schizont *(C)* and late schizont *(D)* expressing a red fluorescent mitochondrion marker protein and a green fluorescent apicoplast marker protein.

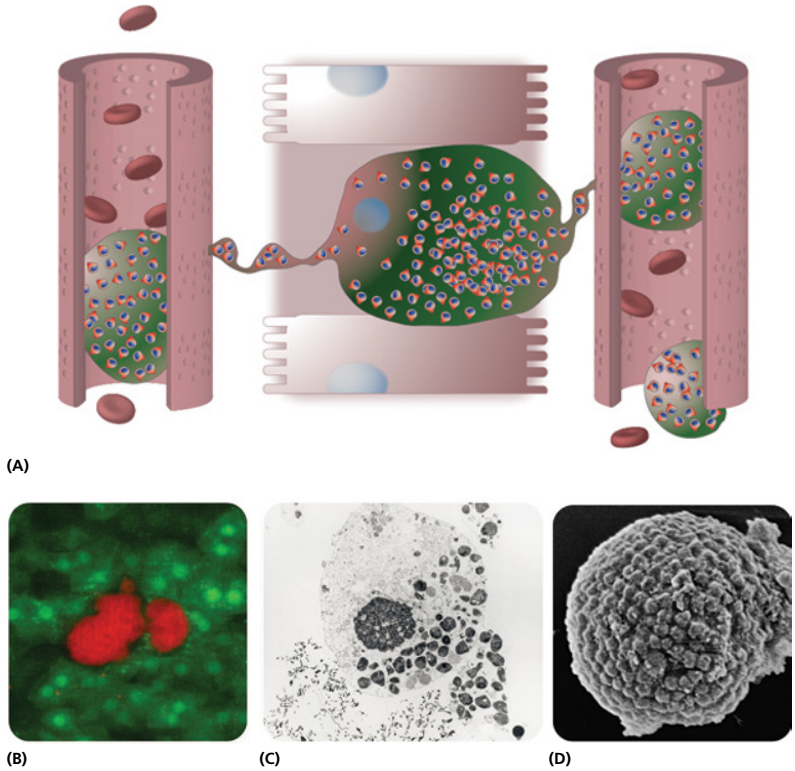

Figure 2.6 Merosome formation *in vivo* and *in vitro:* The scheme depicts the events surrounding merosome formation *in vivo*. *A,* Upon PVM rupture, the host cell detaches from the neighboring cells; vesicles (merosomes) bud off and are constantly filled with infectious merozoites. *B,* Intravital image of mCherry-expressing parasites infecting an LC3-GFP-expressing transgenic mouse. *C,* TEM of a detached cell with a budding merosome. *D,* REM image of a merosome filled with many mature merozoites.

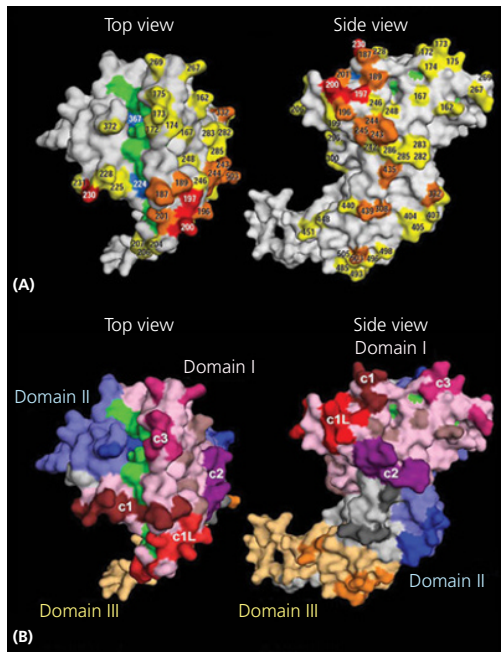

Figure 3.7 Structure of apical membrane antigen 1. Polymorphic amino acids shown on the apical membrane antigen 1 (AMA1) crystal structure. Polymorphisms are based on sequence data from *P. falciparum* infections acquired at a vaccine testing site in Mali, West Africa. *A,* Polymorphic residues are numbered and highlighted. *Yellow* and *blue* residues are dimorphic, *orange* residues are trimorphic, and *red* residues have four to six possible amino acids. Residues highlighted in *green* and *blue* make up the hydrophobic pocket hypothesized to be a binding site between AMA1 and the rest of the erythrocyte invasion machinery, with *blue* indicating polymorphic residues within the pocket. *B,* Conserved residues in AMA1 domains I, II, and III are highlighted, respectively, in *light pink, light blue,* and *light orange.* Polymorphic residues in domain I are highlighted in *dark brown* (c1), *red* (c1 and c1L), *purple* (c2), *dark pink* (c3), and *light brown* (not incorporated in a cluster). Polymorphic residues in domains II and III are highlighted, respectively, in *dark blue* and *dark orange. Light gray* residues are not part of any of the three major domains, and *dark gray* residues are polymorphisms within the interdomain region. *Source:* Takala SL, Coulibaly D, Thera MA, Batchelor AH, Cummings MP, *et al.* 2009. Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. *Science Translational Medicine.* 1:2ra5.

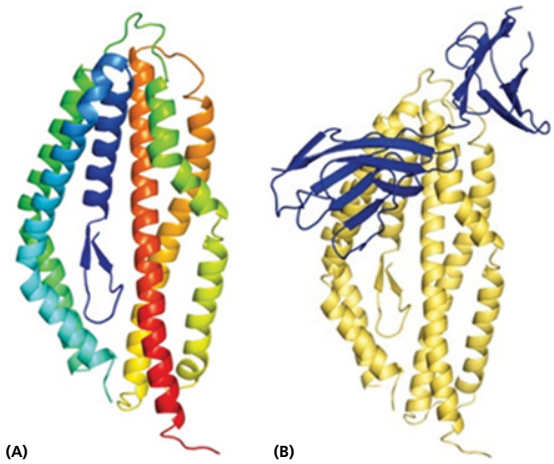

Figure 3.11 Three-dimensional structure of PfRH5 and basigin. *A,* Crystal structure of the PfRH5 protein. *B,* The structure of PfRH5 (depicted in *yellow*) bound to basigin (depicted in *blue*). *Source:* Wright KE, Hjerrild KA, Bartlett J, Douglas AD, Jin J, *et al.* 2014. Structure of malaria invasion protein RH5 with erythrocyte basigin and blocking antibodies. *Nature.* 515(7527):427–430.

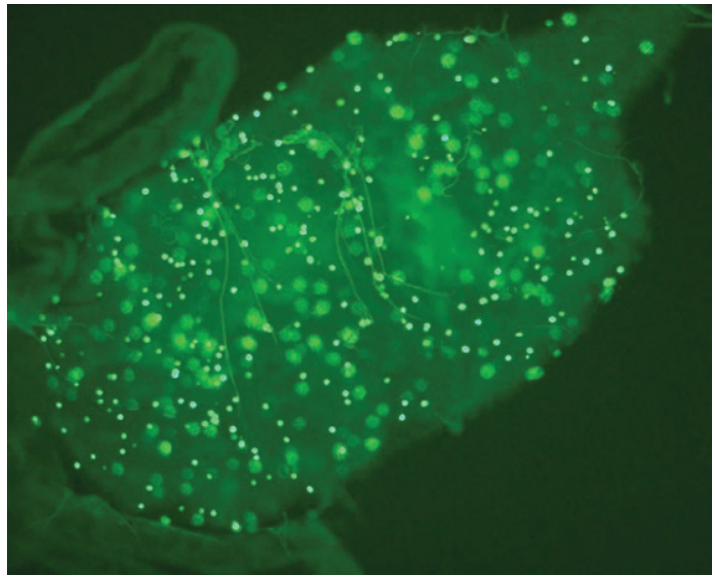

Figure 4.8 Image of dissected midgut of *Anopheles stephensi* infected 5 days previously with a GFP-expressing clone of *Plasmodium berghei.* Note that oocysts on one side of the gut appear small (in focus) and on the other side large (out of focus). Oocyst numbers in such images are readily counted by simple algorithms. (Image from Delves and Sinden, 2010.)

Figure 4.12 3D reconstruction of anterior pole from cryoelectron tomographs of sporozoite (cf. ookinetes, Fig. 4.3a). Purple, plasma membrane; green, microtubules; yellow, inner membrane complex; pink, rhoptry; blue, micronemes; brown, polar rings (MTOC). (Image from Kudryashev *et al.*, 2012.)

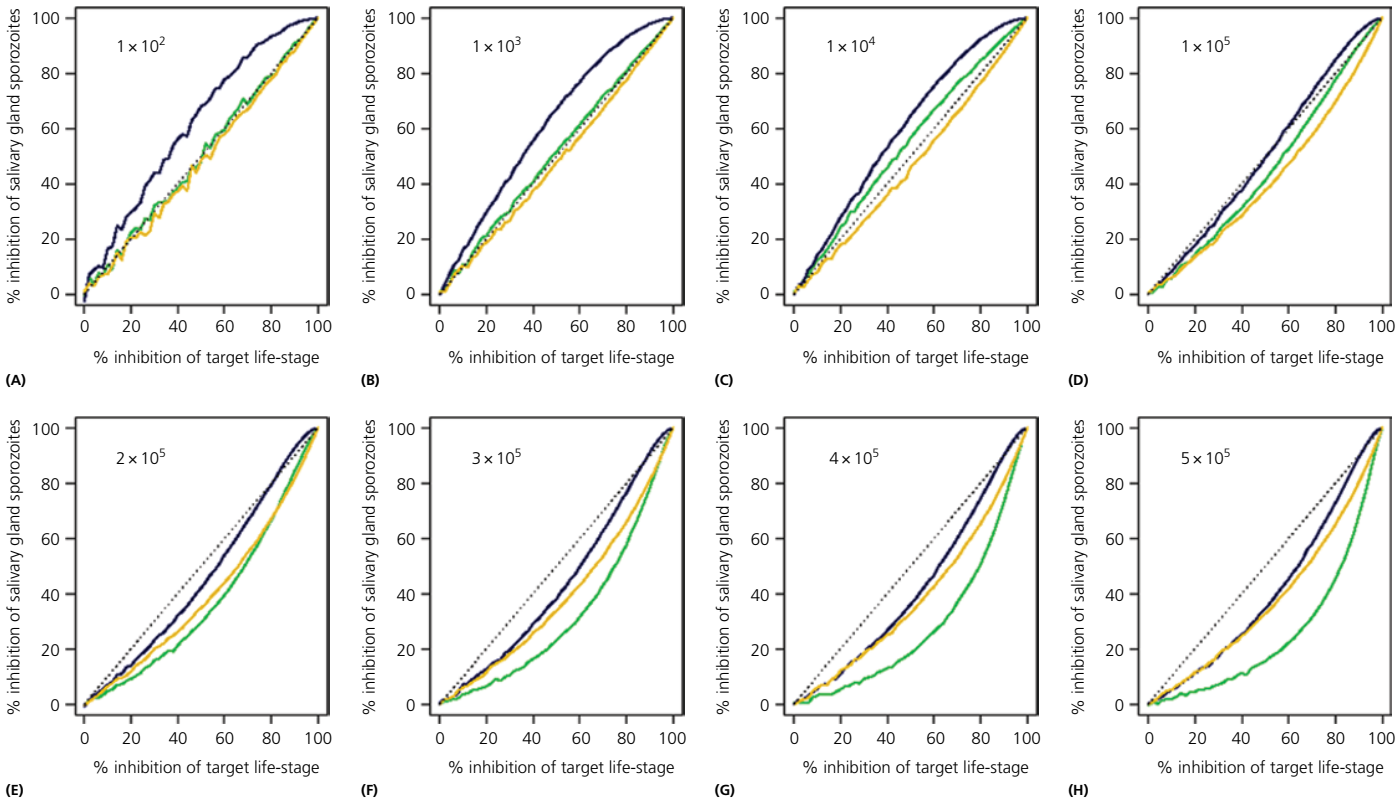

Figure 4.18 The theoretical relationships between the reductions that could be achieved by a transmission blocking intervention in oocyst (yellow), ookinete (blue), or macrogametocyte (green) numbers and the corresponding reductions that would ensue in salivary gland sporozoite number. Note how this relationship is markedly affected by the initial challenge infection (described as macrogametocyte number, top left corner of each box A–H) offered to the mosquito. (Image from Sinden, 2010.)

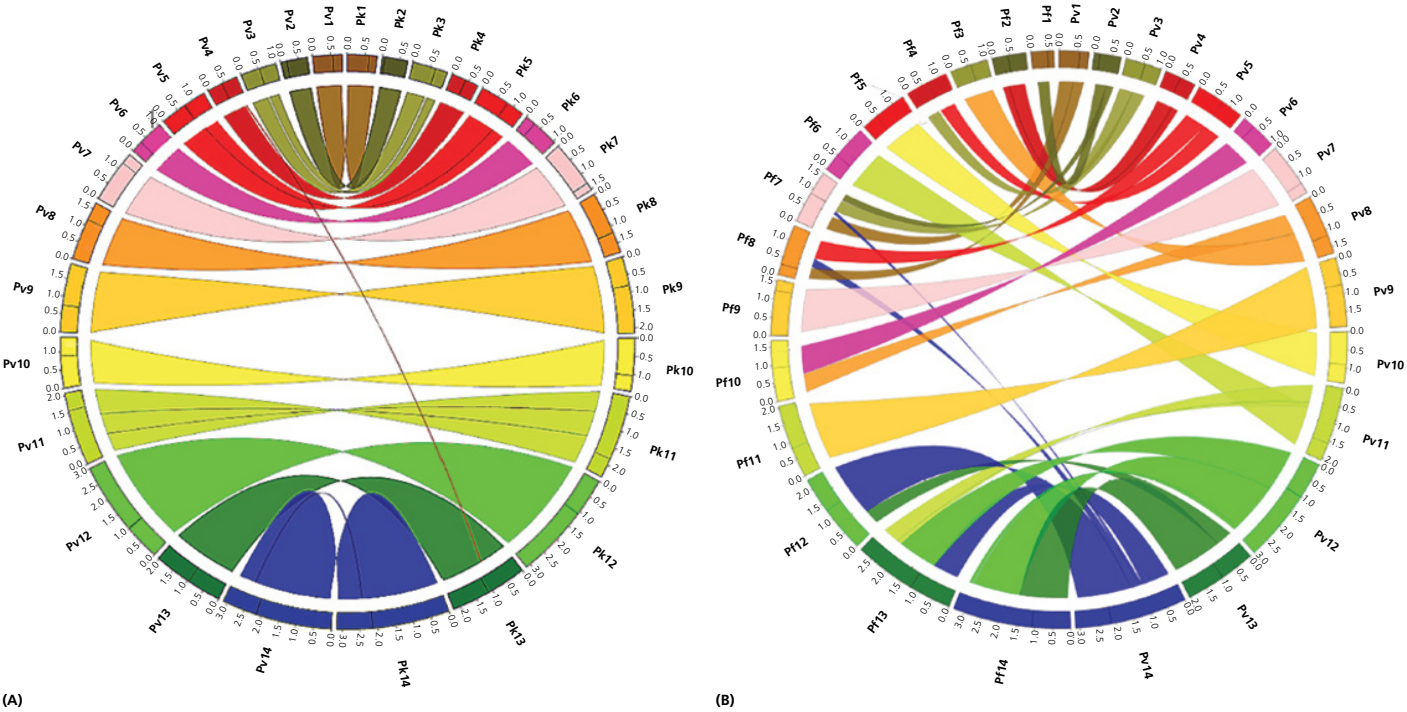

Figure 5.2 Synteny map of the *Plasmodium vivax* genomes as compared to *(A)* a closely related species, *Plasmodium knowlesi,* and *(B)* a more distantly related species, *Plasmodium falciparum*. Each block of color on the periphery of the circles represents a chromosome. Pk, *Plasmodium knowlesi*; Pv, *Plasmodium vivax;* Pf, *Plasmodium falciparum*. These figures show a larger number of rearrangements that have occurred over a greater amount of evolutionary time. They also show that the rearrangements do not occur as single genes, but as large "synteny blocks." (Adapted from Frech and Chen 2011.)

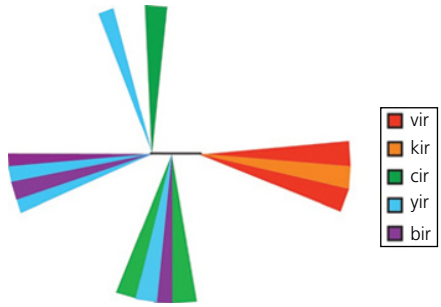

Figure 5.3 Evolutionary relatedness of genes of the *pir* families. Each wedge represents a group of paralogues from each different subfamily of *pir* genes. Note that the primate parasite genes group together, the *virs* (from *P. vivax*) and *kirs* (from *P. knowlesi*) group separately from the rodent parasite genes; however, they do not form a single group by species. Similarly, some of the rodent *pirs* group by species relatedness (a group of *yirs* from *P. yoelii* and *birs* from *P. berghei*), shown in Figure 5.1. Other rodent *pirs* group all together. This shows that the defining characteristics of the rodent and primate *pir* paralogues originated after the split of these two larger groups (see Figure 5.1), whereas within the groups many paralogues retain older characteristics from before the species split.

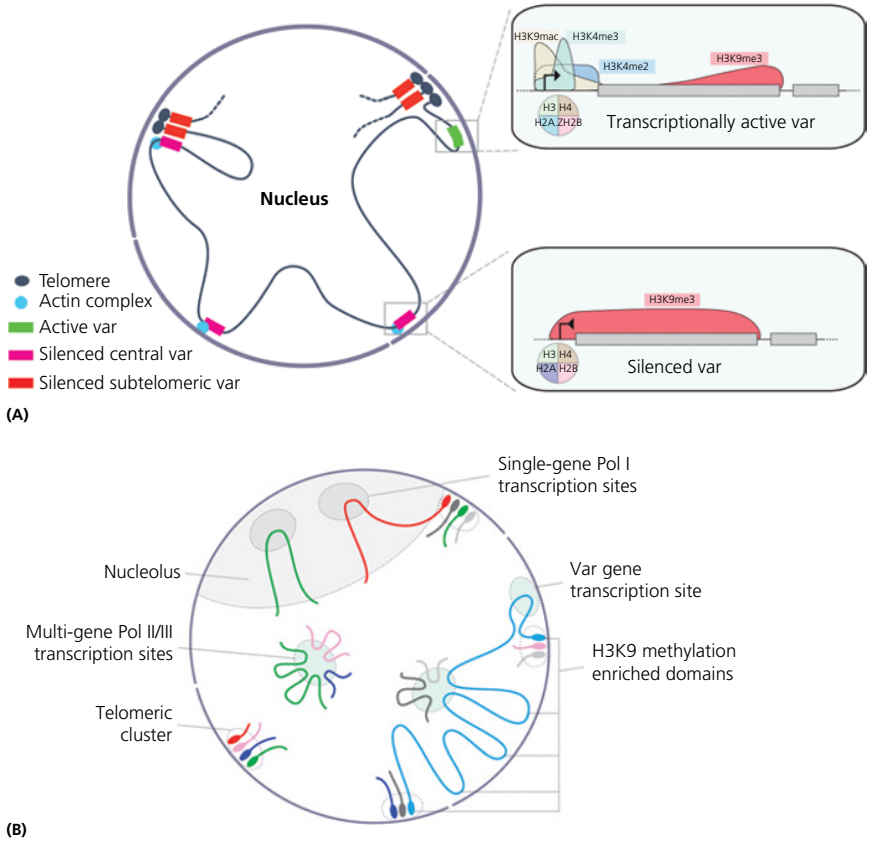

Figure 6.2 A) Schematic representation of histone marks and histone variants linked with silent and active *var* gene loci. A similar situation applies to other clonally variant gene families. The spatial organization of the *var* gene family in the nucleus is also illustrated. B) Model for the nuclear organization of *P. falciparum* genomic DNA at the ring stage indicating the major nuclear compartments: nucleolus, Pol I and Pol II/III transcription sites, telomeric clusters, and chromosome organization. (Adapted from Mancio-Silva 2010.)

Domain ID	Gene ID_PlasmoDB Version 9	Motif	Expression profile
PFE0840c_D2	PF3D7_0516800	GACATC	
PFF0670w_D1	PF3D7_0613800	TAAGCC	
PFF0670w_D2	PF3D7_0613800	CTCTAG	
PFF0200c_DLD	PF3D7_0604100	GTGCAC	
MAL8P1.153	PF3D7_0802100	ACACAC	
PF11_0091	PF3D7_1107800	GCATAC	
PFD0985w_D1	PF3D7_0420300	GTGTGT	
PFD0985w_D2	PF3D7_0420300	TGTTAC	
PF11_0442	PF3D7_1143100	GCTAGC	
PF10_0075_D1	PF3D7_1007700	GTCGAC	
PF10_0075_D2	PF3D7_1007700	TCTTGC	
PF10_0075_D3	PF3D7_1007700	GTGCAC	
PFL1075w	PF3D7_1222400	TATATA	
PFL1900w_DLD	PF3D7_1239200	TCTACA	
PF14_0533	PF3D7_1456000	CACACA	
PF13_0097	PF3D7_1317200	AGCTCA	
PF11_0404_D1	PF3D7_1139300	AGAACA	
PF13_0235_D1	PF3D7_1342900	GCCCCG	
PF13_0267	PF3D7_1350900	CTAGAA	
PF07_0126_DLD	PF3D7_0730300	ATTTTCC	
PF14_0079	PF3D7_1408200	GCAACC	
PF14_0633	PF3D7_1466400	GCATGC	

Figure 8.1 Overview of transcription profiles of the ApiAp2 genes. The figure shows a list of AP2 domains of 20 ApiAP2 proteins, which are predicted to bind with DNA motifs in *P. falciparum* (Campbell 2010). The name of the AP2 domains, corresponding PlasmoDB gene ID, primary motif recognized, and IDC transcriptional profile are listed in the first, second, third, and fourth columns, respectively. D1, D2, and D3 refer to the domain number on the protein, and DLD refers to two domains (domain linker domain). The transcriptional profiles shown are taken from a previous study (Foth 2011), where microarrays were carried out at 24 time points across the 48-hour IDC.

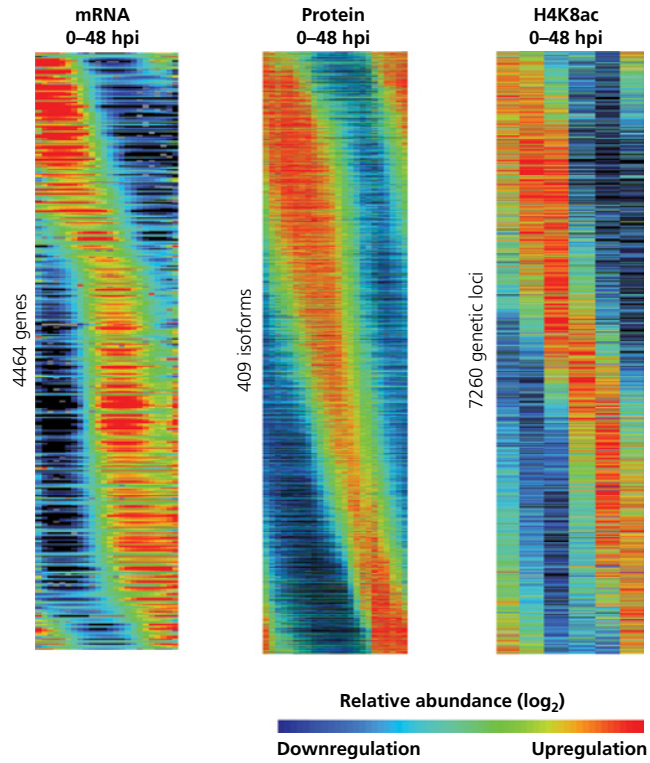

Figure 8.2 Overview of the expression, protein, and histone modification profiles during *P. falciparum* IDC. The figure depicts abundance profiles of 4464 transcripts (corresponding to 4464 genes), 409 protein isoforms (corresponding to 149 genes), and 7260 acetylated histone H4 lysine 8 (H4K8ac) profiles (corresponding to 3108 genes) across the IDC in *P. falciparum*. The results are based on oligonucleotide microarray for mRNA or histone-modification profiles and 2D-DIGE for protein profiles and show the prevalence of single peak profiles across the IDC in each case. The heat maps depicting mRNA (on the left), protein (in the middle) and H4K8ac (on the right) abundance profiles comprise 24, 24, and 6 time points, respectively, covering 0 to 48 hours after invasion (hpi). Note that the rows in each heat map representing transcripts, proteins, and H4K8ac *do not* correspond to one another. Each row represents the transcript-abundance profile for each gene, protein-abundance profile for each protein isoform, and H4K8ac-abundance profile for each genetic locus as seen in left, middle, and right panels, respectively. The abundance profiles were sorted according to their Fourier phase, and the color scale represents the lowest smoothed profiles calculated from centered curves of relative log2 occupancy ratios. Data for this figure were derived from 2 previous studies (Gupta 2013; Foth 2011).

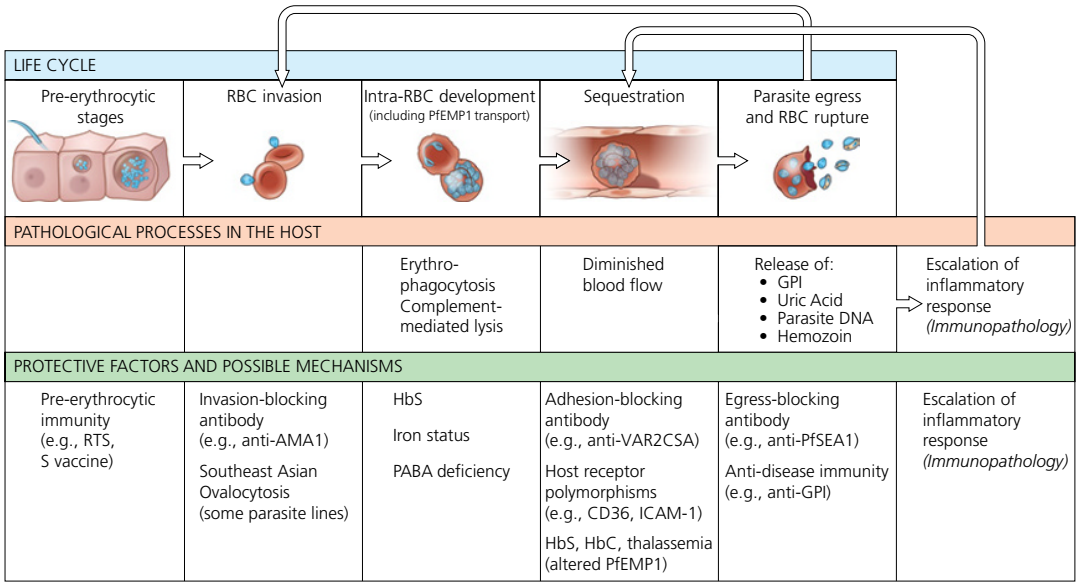

Figure 16.2 Pathological processes implicated in the pathogenesis of malarial disease are associated with specific phases of parasite development. Specific factors that reduce the risk of severe malaria are thought to affect these pathological processes or to prevent the initial infection that can result in severe malaria. These factors often form the conceptual basis for adjunctive therapies to reduce mortality after severe malaria develops or for vaccines to prevent the development of severe malaria.

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