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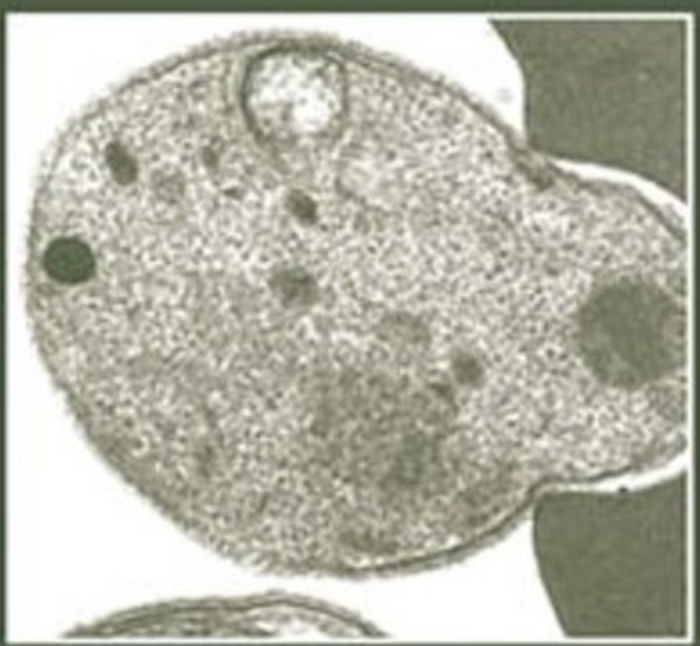

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CHAPTER ONE

Transgenic Mouse Models of Angiogenesis and Lymphangiogenesis

Domenico Ribatti

Contents

Abstract

The development of transgenic technologies in mice has allowed the study of the consequences of genetic alterations on angiogenesis and lymphangiogenesis. This review summarizes the murine models currently available for studies involving the manipulation of angiogenesis and lymphangiogenesis. Abnormal embryonic vascular development, resulting from defects in the formation of a primitive vascular plexus, has been observed in mice lacking vascular endothelial growth factor, vascular endothelial growth factor receptor-1 and -2, transforming growth factor-β, fibronectin, or vascular endothelial cadherin. Defects in the expansion and remodeling of the embryonic vasculature occur in mice deficient in Tie-1, Tie-2, or angiopoietin-1, and in mice overexpressing neuropilin or angiopoietin-2. Impaired recruitment and investment of mural cells have been observed in mice with disruption of the genes encoding platelet-derived growth factor-B, platelet-derived growth factor-B receptor, and tissue factor.

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Gene-targeting experiments in mice have identified the EphB/ephrinB system as a critical and rate-limiting determinant of arteriovenous differentiation during embryonic vascular development. Vascular endothelial growth factor-C is necessary for the initial sprouting and migration of lymphatic endothelial cells from embryonic veins, and mice lacking vascular endothelial growth factor-C die prenatally, whereas vascular endothelial growth factor-D is dispensable for embryonic lymphatic development.

Key Words: Angiogenesis, Endothelium, Lymphangiogenesis, Transgenic mouse models, Vascular system.

1. Introduction

1.1. Angiogenesis

Embryonic vascular development involves a complex series of events during which endothelial cells differentiate, proliferate, migrate, and undergo maturation into an organized vascular network (Risau and Flamme, 1995; Risau, 1997). The first step in vessel development is called vasculogenesis, in which mesoderm-derived angioblasts proliferate and organize into the primitive vascular plexus, consisting of the major vessels (Fig. 1.1). Remodeling and expansion of these primary vessels through both pruning and vessel enlargement, which result in a closely interconnecting branching pattern, is called angiogenesis, that is, sprouting of new vessels from existing ones

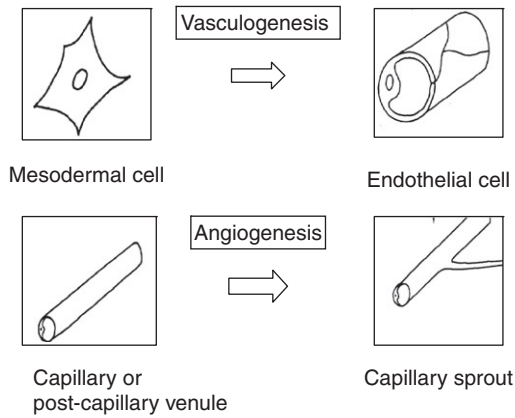

Figure 1.1 Vasculogenesis and angiogenesis. Two interrelated but separable processes are responsible for the formation of new capillaries: vasculogenesis, the *in situ* differentiation of mesodermal precursor cells into endothelial cells, which subsequently form a primary capillary plexus, and angiogenesis, the formation of new vessels by a process of sprouting from preexisting vessels.

(Figs. 1.1 and 1.2). The loops between vessels can also form via another mechanism called intussusceptive growth, a type of angiogenesis involving the *in situ* remodeling of vessels by protruding interstitial tissue columns. In this process, a large sinusoidal capillary is divided into smaller capillaries, which then grow separately (Burri and Tarek, 1990). According to intussusceptive growth, the capillary network increases its complexity and vascular surface by inserting of a multitude of transcapillary pillars, through four consecutive steps: (1) creation of a zone of contact between opposite capillary walls; (2) reorganization of the intercellular junctions of the endothelium, with central perforation of the endothelial bilayer; (3) formation of an interstitial pillar core; and (4) subsequent invasion of the pillar by cytoplasmic extensions of myofibroblasts and pericytes, and by collagen fibrils (Fig. 1.3). It is thought that the pillars then increase in diameter and become a capillary mesh.

Angiogenesis plays a pivotal role during embryonal development and later, in adult life, in several physiological (e.g., corpus luteum formation)

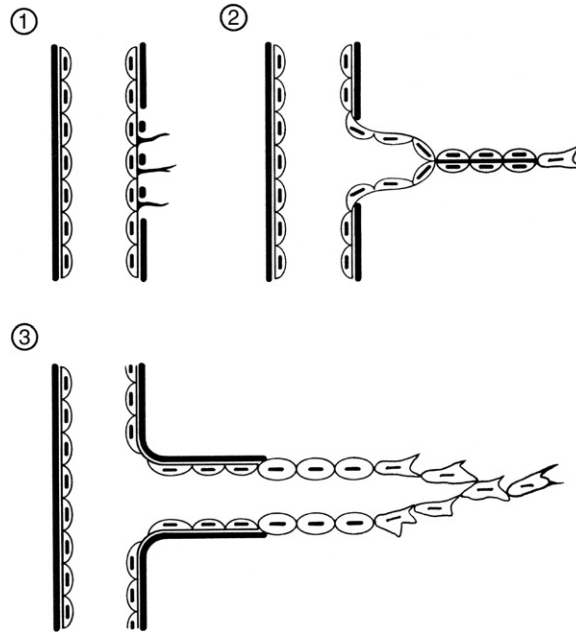

Figure 1.2 Schematic representation of the angiogenic process. (1) In response to an angiogenic stimulus, endothelial cells lining an existing capillary are activated. They then degrade the underlying basement membrane and extend thin cytoplasmic-processes in the direction of the stimulus. (2) The cells then migrate into the surrounding matrix, within which they form a capillary sprout. Lumen formation commences in the proximal part of the sprout. (3) Sprout maturation is completed by reconstitution of the basement membrane and the formation of a patent capillary lumen.

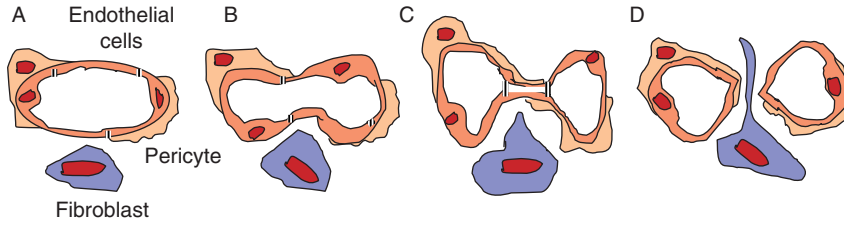

Figure 1.3 Schematic representation of the process generating new vessels by intussusceptive growth. The process is characterized by a progressive (A–C) protrusion of opposing capillary walls into the vessel lumen. Finally, as shown in (D), the endothelial cells are perforated centrally and the newly formed pillar is invaded by fibroblasts and pericytes.

and pathological conditions, such as tumor formation and chronic inflammation, in which angiogenesis itself may contribute to the progression of disease (Folkman, 1995). Under physiological conditions, angiogenesis depends on the balance of positive and negative angiogenic modulators within the vascular microenvironment (Hanahan and Folkman, 1996) and requires the functional activities of a number of molecules, including angiogenic factors, extracellular matrix proteins, adhesion receptors, and proteolytic enzymes. As a consequence, angiogenic endothelial cells have a distinct gene expression pattern that is characterized by a switch of the cell proteolytic balance toward an invasive phenotype as well as by the expression of specific adhesion molecules (Pepper *et al.*, 1996). Angiogenic factors are potent growth factors that promote proliferation and differentiation of endothelial cells. The major angiogenic and antiangiogenic factors are listed in Table 1.1.

The endothelial layer of the perineural and yolk sac vascular plexuses, anterior cardinal veins, major trunk vessels, and vessels that perfuse organs with endoderm-derived epithelial layers form by vasculogenesis. In contrast, internal carotid arteries, trunk intersomitic vessels, and vessels that perfuse organs with ectoderm-derived epithelial cells form primarily by angiogenesis.

1.2. Lymphangiogenesis

The lymphatic system is implicated in interstitial fluid balance regulation, immune cell trafficking, edema, and cancer metastasis. However, the sequence of events that initiate and coordinate lymphangiogenesis remains elusive. In effect, the understanding of physiological regulation of the lymphatic vasculature has been overshadowed by the greater emphasis focused on angiogenesis, and delayed by a lack of specific markers, thereby limiting this field to no more than a descriptive characterization.

Table 1.1 Endogenous Angiogenic and Antiangiogenic Factors That Regulate Angiogenesis

Factor	Reference
Angiogenic	
Vascular endothelial growth factor (VEGF)	Ribatti, 2005
Fibroblast growth factor-2 (FGF-2)	Presta *et al.*, 2005
Placental growth factor (PlGF)	De Falco *et al.*, 2002
Platelet derived growth factor (PDGF)	Armulik *et al.*, 2005
Transforming growth factors (TGF-α and -*β*)	Bertolino *et al.*, 2005
Epidermal growth factor (EGF)	Nezu *et al.*, 1992
Hepatocyte growth factor (HGF)	Rosen *et al.*, 1997
Platelet-activating factor (PAF)	Montrucchio *et al.*, 2000
Tumor necrosis factor-α (TNF-α)	Leibovich *et al.*, 1987
Insulin-like growth factor (IGF)	Delafontaine, 1995
Angiogenin	Tello-Montoliu *et al.*, 2006
Angiopoietin-1	Tsigkos *et al.*, 2003
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Bikfalvi and Han, 1994
Granulocyte colony-stimulating factor (G-CSF)	Bikfalvi and Han, 1994
Erythropoietin	Ribatti *et al.*, 2003
Interleukin-6	Nilsson *et al.*, 2005
Interleukin-8	Koch *et al.*, 1992
Antiangiogenic	
Thrombospondin	Iruela-Arispe *et al.*, 2004
Angiostatin	Cao and Xue, 2004
Endostatin	Folkman, 2006
Interferon-α	Ezekowitz *et al.*, 1992
Interleukin-12	Airoldi *et al.*, 2007
Angiopoietin-2	Tsigkos *et al.*, 2003
Tissue inhibitors of metalloproteinases	Handsley and Edwards, 2005

New insights into lymphangiogenesis have been due to the discovery of lymphatic-specific markers and growth factors of the vascular endothelial growth factor (VEGF) family, such as VEGF-C and VEGF-D.

VEGF-C is a VEGF isoform closely related to VEGF-D, characterized by the presence of unique amino- and carboxy-terminal extensions flanking the common VEGF homology domain (Joukov *et al.*, 1996). VEGF-C may play several functions in the organization of the vascular tree. VEGF-C

induces lymphangiogenesis in the ears of mice and in the chick embryo chorioallantoic membrane (CAM) (Enholm *et al.*, 2001; Oh *et al.*, 1997), and lymphatic vessel enlargement in the skin (Jeltsch *et al.*, 1997). VEGF-C also has potent effects on blood vessels because its fully processed form also binds to VEGF receptor (VEGFR)-2 of blood vessels and stimulates angiogenesis (Cao *et al.*, 1998).

VEGF-D is angiogenic in the rabbit corneal assay (Marconcini *et al.*, 1999). In a mouse tumor model, VEGF-D promoted lymphangiogenesis (Achen *et al.*, 1998) and metastatic spread via the lymphatics (Stacker *et al.*, 2001). Lymphatic spread was blocked by a VEGF-D-specific antibody. Achen *et al.* (2002) analyzed VEGF-D activity in human tumors and a mouse model of metastasis. Tumor vessels positive for VEGF-D were also positive for VEGFR-2 and/or VEGFR-3 but negative for VEGF-D mRNA, indicating that VEGF-D is secreted by tumor cells and subsequently associates with the endothelium via receptor-mediated uptake. In the mouse model, VEGF-D synthesized in tumor cells became localized on the endothelium and thereby promoted metastatic spread. Overall data indicate that VEGF-D promotes tumor angiogenesis, lymphangiogenesis, and metastatic spread by a paracrine mechanism.

2. Factors Involved in Abnormal Embryonic Vascular and Lymphatic Development

Gene deletion studies have led to the identification of numerous genes that are critical to angiogenesis and lymphangiogenesis (Fig. 1.4). In all these studies embryonic vascular defects have been attributed to aberrant angiogenesis, failed remodeling of primary vascular networks, or impaired mural cell investment.

The first blood vessels of the mouse begin to form in the yolk sac on day 6–6.5 (Drake and Fleming, 2000). The extraembryonic mesoderm of the yolk sac gives rise to blood and endothelial cells, which begin to form morphologically identifiable "blood islands." Primitive hematopoiesis, the formation of embryonic blood cells, and vasculogenesis are therefore closely associated processes in the yolk sac and may arise from a common progenitor, the "hemangioblast." Later in development (i.e., on day 7–7.5), vasculogenesis is initiated within the embryo proper, with blood vessels appearing in the following order: endocardium, primary vascular networks lateral to the midline, paired dorsal aortas, and head and cardinal vessels (Fig. 1.5).

It is of central importance to understand that nascent blood vessels in one region of a mutant embryo can appear abnormal, whereas those in regions that were initiated at a later stage are normal.

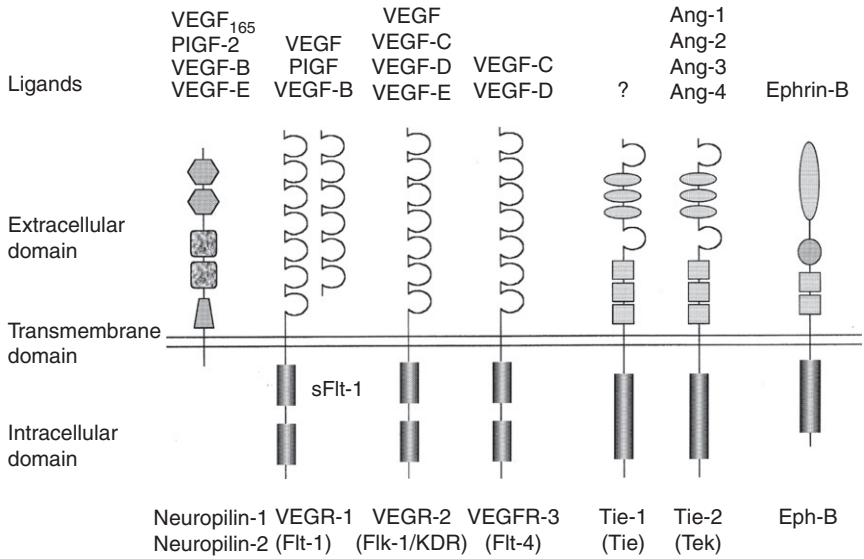

Figure 1.4 Endothelial growth factor receptors and their ligands involved in vasculogenesis, angiogenesis, and lymphangiogenesis. Ang, angiopoietin; KDR, kinase insert domain receptor; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR vascular endothelial growth factor receptor.

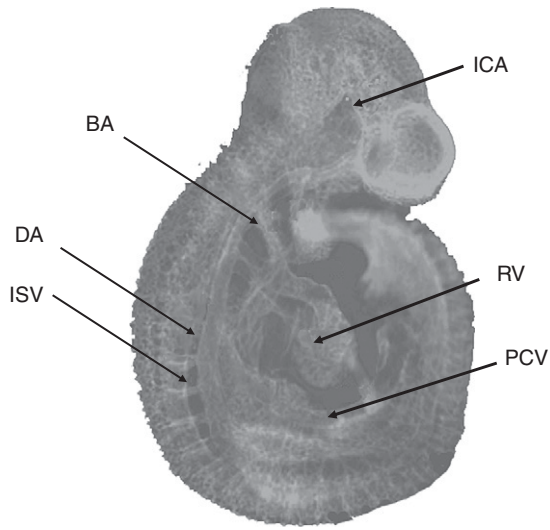

Figure 1.5 Embryonic vasculature of a mouse embryo on day 9.5 of development. BA, branchial arteries; DA, dorsal aorta; ICA, intercarotid artery; ISV, intersomitic vessels; PCV, posterior cardinal vein; RV, right ventricle.

2.1. VEGF family

The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF). The VEGF gene encodes VEGF-A isoforms (VEGF-$A_{121-206}$) by alternative splicing that differently encodes exons 6 and 7, where the peptides responsible for the heparin-binding capacity are located. The heparin-binding domains help VEGF-A to anchor to the extracellular matrix and are involved in binding to heparin sulfate and presentation to VEGF receptors. VEGF isoforms with higher heparin affinity are rapidly sequestrated by the heparan sulfate proteoglycans located at the endothelial cell surface and in the extracellular matrix.

All the VEGF isoforms share common tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3) (Fig. 1.6). All three receptors have a similar overall structure, with a ligand-binding extracellular domain containing seven immunoglobulin-like repeats and an intracellular domain comprising a juxtamembrane sequence, tyrosine kinase domain with kinase insert, and carboxy-terminal tail. VEGF-A binds with high affinity to VEGFR-1 and VEGFR-2 and plays an essential role in vasculogenesis and angiogenesis. It has also been shown to induce lymphangiogenesis through VEGFR-2. VEGF-B overlaps VEGF-A activities by activating VEGFR-1. VEGF-C and VEGF-D are both angiogenic via VEGFR-2 and VEGFR-3 and lymphangiogenic (primarily VEGF-D) via VEGFR-3.

2.1.1. VEGF-A

VEGF-A-deficient mouse embryos die between days 8.5 and 9.5 postcoitum and exhibit severe phenotypes similar to that of VEGFR-$2^{-/-}$ and VEGFR-$2^{+/-}$ embryos (Carmeliet *et al.*, 1996a; Ferrara *et al.*, 1996).

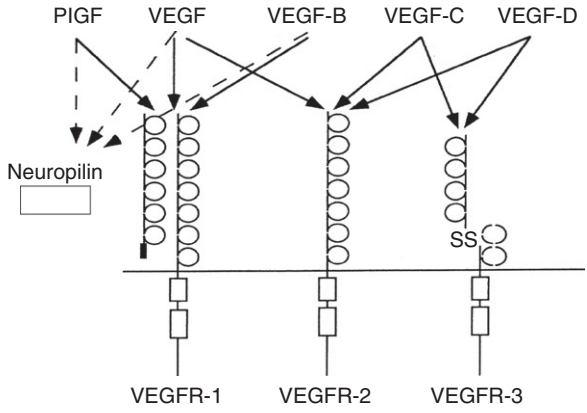

Figure 1.6 VEGFR interactions and signaling responses.

As a consequence of the targeted inactivation of a single allele embryos died between 11 and 12 days, the dorsal aorta had a smaller lumen, angiogenic sprouting of vessels was reduced, development of large thoracic blood vessels appeared abnormal, and only an irregular plexus of enlarged capillaries was present in the yolk sac and placenta.

The lethality resulting from the loss of a single allele is indicative of tight dose-dependent regulation of embryonic vessel development by VEGF-A. Loss of VEGF-A does not prevent initial differentiation of angioblasts, but precise VEGF-A concentration gradients are required for correct lumen formation, sprouting, and angiogenesis. The vascular phenotype observed in the VEGF knockout mouse is considerably less severe than that observed in the VEGFR-2 knockout mouse, suggesting that other VEGFR-2 ligands may be able to compensate to some extent for the loss of VEGF.

Mice expressing the VEGF-A_{120} isoform alone (VEGF-$A^{120/120}$ mice) were generated using the Cre–*loxP* system to remove exons 6 and 7, which encode basic domains that are present only in VEGF-A_{164} and/or $VEGF_{188}$ (Carmeliet *et al.*, 1999a). A fraction of homozygous VEGF-A_{120} embryos died shortly after birth. The remainder of these mutant mice gained less weight and died before postnatal day 14 of cardiac failure, exhibiting depressed myocardial contractility and cardiac dilatation. Whereas in wild-type mice the number of capillaries and coronary vessels increased 3- and 10-fold during the first three postnatal weeks, the capillary density did not change in VEGF-$A^{120/120}$ mice, resulting in greater intercapillary distances, increased myocyte-to-capillary ratios, and impaired oxygen delivery. VEGF-$A^{120/120}$ hearts also contained fewer coronary vessels and reduced smooth muscle coverage, and the vascular defects resulted in myocardial ischemia.

VEGF-A continues to be critical during early postnatal growth and development, as evidenced by postnatal VEGF-A inactivation using Cre–*loxP*-mediated VEGF-A gene deletion, or by administration of a soluble VEGFR that blocks VEGF-A action (Gerber *et al.*, 1999a). VEGF-A inactivation in older animals is much less traumatic, seemingly affecting only those structures that continue to undergo vascular remodeling such as bone growth plates or ovarian corpus luteum (Ferrara *et al.*, 1998; Gerber *et al.*, 1999b).

Leppanen *et al.* (2006) have generated a transgenic mouse in which human (h)VEGF-A_{165} expression has been silenced with a *loxP*–stop fragment, and they have used this model to study the effects of hVEGF-A_{165} overexpression in mice after systemic adenovirus-mediated Cre gene transfer. This experimental model leads to the expression of hVEGF-A_{165} in only a low number of cells in the target tissues in adult mice. Most mice were healthy without any major consequences up to 10 months after the activation of hVEGF-A_{165} expression. However, one mouse with a high plasma hVEGF-A_{165} level died spontaneously because of bleeding into the

abdominal cavity and liver hemangioma, and two mice developed malignant tumors (hepatocellular carcinoma and lung adenocarcinoma), which were not seen in control mice.

2.1.2. VEGF-B

Mice lacking VEGF-B are overtly normal and fertile, but their hearts are reduced in size and display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial ischemia, suggesting that VEGF-B may have a role in coronary vascularization and growth (Bellomo *et al.*, 2000).

2.1.3. VEGF-C and VEGF-D

VEGF-C knockout mice died because of the lack of lymphatic vessels, whereas VEGF-$C^{+/-}$ mice survive despite defects in the lymphatic vessels (Karkkainen *et al.*, 2001, 2004). VEGF-C knockout mice failed to form primary lymph sacs, lack all lymphatic vessels, develop severe edema, and die before birth. Even the loss of a single VEGF-C allele in heterozygous mutants leads to lymphatic vessel hypoplasia and lymphoedema in the skin. VEGF-D-deficient mice are viable (Baldwin *et al.*, 2005). VEGF-C is necessary for the initial sprouting and migration of lymphatic endothelial cells from embryonic veins, whereas VEGF-D is dispensable for embryonic lymphatic development.

Transgenic overexpression of VEGF-C leads to lymphatic hyperplasia (Olofsson *et al.*, 1999). Studies with transgenic mice overexpressing VEGF-C and VEGF-D under the control of keratinocyte- or pancreas-specific promoters have demonstrated the role of these growth factors, mainly in lymphangiogenesis (Jeltsch *et al.*, 1997; Mandriota *et al.*, 2001).

2.1.4. VEGFR-1

Loss of VEGFR-1 results in embryonic lethality around day 10 of gestation: despite the presence of numerous differentiated endothelial cells, the latter failed to form an organized vascular network and assembled in abnormally large and fused vessels (Fong *et al.*, 1995). The knockout phenotype was characterized by irregular lacunar sinusoids in the yolk sac and endothelial cells inside the vessel lumen. All the major vascular structures inside the embryo and in the yolk sac were affected. These findings suggest a possible role of VEGFR-1 in contact inhibition of endothelial cell growth or in endothelial cell assembly. The phenotype is more suggestive of an overgrowth of endothelial cells than a loss of cells, as confirmed by chimera studies (Fong *et al.*, 1999). Vascular disorganization in the mutant embryos seems to result from an increase in the number of endothelial cells and their progenitors, which is attributable primarily to altered fate of mesenchymal cells, rather than increased proliferation or reduced apoptosis of progenitors (Fong *et al.*, 1999).

2.1.5. VEGFR-2

Mice deficient in VEGFR-2 (VEGFR-2$^{-/-}$) died *in utero* between days 8.5 and 9.5 postcoitum, as a result of an early defect in the development of hematopoietic cells and endothelial cells, and a complete lack of vasculature. Yolk sac blood islands were absent at 7.5 days, organized blood vessels could not be observed in the embryo or yolk sac at any stage, and hematopoietic progenitors were absent (Shalaby *et al.*, 1995). Analysis of chimeric VEGFR-2-deficient mice indicated a cell-autonomous requirement for VEGFR-2 in endothelial cell differentiation (Shalaby *et al.*, 1997).

Whereas mice heterozygous for the VEGFR-2 deletion developed normally, homozygous knockout embryos formed only a few blood vessels, and no differentiated endothelial or endocardial cells. Furthermore, VEGFR-2-deficient cells were unable to contribute to the vascular network when placed in wild-type hosts, instead accumulating in ectopic locations, which suggests that VEGFR-2 is required for reception of a signal directing angioblasts to their proper position (Shalaby *et al.*, 1997).

An integrative model has been proposed in which the helix–loop–helix (HLH) transcription factor Tal-1 (or Scl) in combination with VEGFR-2 determines hemangioblast formation and differentiation (Ema *et al.*, 2003). Tal-1 expression in VEGFR-2 mesodermal precursors would favor proliferation of VEGFR-2^{+}Tal-1^{+} hemangioblasts, and cells expressing high levels of Tal-1 would differentiate into endothelial cells, whereas those expressing lower levels differentiate into smooth muscle cells.

2.1.6. VEGFR-3

During mouse embryogenesis VEGFR-3 is first expressed in a subset of vascular endothelial cells on day 7.5 (Dumont *et al.*, 1995; Kaipainen *et al.*, 1995). VEGFR-3-deficient mice died at embryonic day 9.5; loss of VEGFR-3 did not prevent vasculogenesis and angiogenesis sprouting, but impaired remodeling of the expanding embryonic vasculature, suggesting that this receptor is involved in the development of the vascular system before its role in lymphangiogenesis (Dumont *et al.*, 1998). Large vessels were abnormally organized, with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure. Later in embryonic development, when the lymphatic vessels start to sprout at approximately day 10.5, the expression of VEGFR-3 decreased in blood vessels and became restricted almost exclusively to the lymphatic endothelium (Kaipainen *et al.*, 1995).

Studies with transgenic mice overexpressing VEGFR-3 have demonstrated its role mainly in lymphangiogenesis (Veikkola *et al.*, 2001), and when a soluble form of VEGFR-3 was expressed in the skin of transgenic mice, lymphangiogenesis was inhibited and preexisting lymphatics regressed (Makinen *et al.*, 2001).

2.2. Growth factors

2.2.1. Placental growth factor

Placental growth factor (PlGF) enhances angiogenesis only in pathological conditions by displacing VEGF from VEGFR-1, thereby making more VEGF available to bind VEGFR-2; by transmitting angiogenic signals through its receptor VEGFR-1; and by a novel mode of cross-talk, wherein activation of VEGFR-1 by PlGF results in enhanced tyrosine phosphorylation of VEGFR-2, thereby amplifying VEGF-driven vessel growth (Park *et al.*, 1994).

Deficiency of PlGF in transgenic mice did not affect physiological angiogenesis during embryogenesis or neonatal growth but impaired angiogenesis in a variety of pathological conditions. Loss of PlGF impaired angiogenesis, plasma extravasation, and collateral growth during ischemia, inflammation, wound healing, and cancer. Transplantation of wild-type bone marrow rescued the impaired angiogenesis and collateral growth in $PlGF^{-/-}$ mice, indicating that PlGF might have contributed to vessel growth in the adult by mobilizing bone marrow-derived cells. Despite its high expression in the placenta and signaling via VEGFR-1 in trophoblasts, PlGF-deficient mice were fertile (Carmeliet *et al.*, 2001).

2.2.2. Fibroblast growth factor-2

The fibroblast growth factor (FGF) family comprises a large group of about 20 polypeptides. To exert their biological activity, FGFs interact with high-affinity tyrosine kinase FGF receptors (FGFRs). Four members of the FGFR family (FGFR-1, FGFR-2, FGFR-3, and FGFR-4) are encoded by distinct genes and their structural variability is increased by alternative splicing (Presta *et al.*, 2005). Fibroblast growth factor-2 (FGF-2) is one of the best characterized and investigated proangiogenic cytokines (Presta *et al.*, 2005).

When quail blastodisks were explanted and disrupted in culture, the mesoderm cells did not form angioblasts or express proteins associated with endothelial cell lineage (Flamme and Risau, 1992), unless they were treated with FGF-2, in which case blood island-like structures were formed (Flamme and Risau, 1992). Flamme *et al.* (1997) showed that FGF-2 induces pluripotent cells of the quail blastodisk to undergo vasculogenesis and experiments in the chick have suggested that FGF signaling is important for initiation of angioblast specification (Cox and Poole, 2000). FGF-2 has been identified in the chick CAM during vasculogenesis and can promote vessel growth when exogenously added to the CAM during embryo development (Ribatti *et al.*, 1995). Moreover, neutralizing antibodies to FGF-2 inhibited vessel growth when applied locally, suggesting that FGF-2

normally functions to promote vessel growth, possibly by inducing angioblasts from the mesoderm (Ribatti *et al.*, 1995).

FGF-2 knockout mice are morphologically normal (Zhou *et al.*, 1998) and do not show differences in neovascularization following injury (Tobe *et al.*, 1998) or hypoxia (Ozaki *et al.*, 1998). Conversely, transgenic overexpression of FGF-2 does not result in vascular defects, even though an amplified angiogenic response can be observed after wounding or subcutaneous implantation of a Matrigel plug (Fulgham *et al.*, 1999). The apparently normal vascularization in FGF-$2^{-/-}$ mice may reflect the contribution to angiogenesis of several other angiogenic growth factors.

2.2.3. Transforming growth factor-β

The differentiation of progenitor cells into pericytes and smooth muscle cells is promoted by transforming growth factor-β_1 (TGF-β_1) (Armulik *et al.*, 2005). Studies of targeted knockout mice have provided evidence of an essential role for TGF-β_1 signaling in vascular development (Dickson *et al.*, 1995). TGF-$\beta_1^{-/-}$ embryos died on day 10.5 with abnormal development of the yolk sac vasculature. There were dramatic defects in yolk sac vessel development, including incomplete contacts between endothelial and mesothelial layers, leading to distended capillary structures. These mutants showed a range of abnormalities in the extraembryonic vasculature, including delayed vasculogenesis, the development of weak vessels, and even areas in which vessels were entirely absent. Dickson *et al.* (1995) concluded from this work that deficiency in TGF-β_1 resulted in inadequate differentiation of endothelial and hematopoietic cells.

Severe vascular phenotypes have been observed in knockout mice that lack one of the three receptors activin-like kinase-1 (ALK-1), ALK-5, or TGF-βR-2, or endoglin or the downstream effector SMAD5 (Armulik *et al.*, 2005). Embryos deficient for ALK-1 and ALK-5 genes died around days 10.5–11.5 with vascular defects (Oh *et al.*, 2000; Urness *et al.*, 2000; Larsson *et al.*, 2001). TGF-βR-2 mutant embryos died between days 10.5 and 11.5 owing to defects in yolk sac vasculature (Oshima *et al.*, 1996). Mice lacking endoglin also died by day 11.5 with major embryonic and extraembryonic vascular defects, along with defects in endocardial cushion formation and heart development (Arthur *et al.*, 2000; Li *et al.*, 1999). Endoglin knockout mouse exhibited normal vasculogenesis but underwent embryonic lethality as a result of defective vascular remodeling and smooth muscle cell differentiation. Similarly, mutations in endoglin and ALK-1 have been linked to human vascular disorders (hereditary hemorrhagic telangiectasia [HHT]-1 and HHT-2, respectively). The phenotype of embryos lacking SMAD5 was similar to loss of ALK-1, that is, embryonic vessels are enlarged and disorganized (Yang *et al.*, 1999).

2.2.4. Platelet-derived growth factor-B

The platelet-derived growth factor (PDGF) family comprises four family members (e.g., PDGF-A to PDGF-D) that bind, with distinct selectivity, the receptor tyrosine kinases PDGFR-A and PDGFR-B expressed on endothelial cells and smooth muscle cells. PDGF-B is the most characterized member in the PDGF family. Although first discovered as a secretory product of platelets during coagulation, PDGF-B is also expressed in many other cell types, such as endothelial cells, macrophages, smooth muscle cells, fibroblasts, glial cells, neurons, and tumor cells. PDGF-B is secreted by endothelial cells, presumably in response to VEGF, and facilitates recruitment of murals cells (Fig. 1.7). By releasing PDGF, endothelial cells stimulate growth and differentiation of a PDGFR-B-positive progenitor and recruit it around nascent vessels. Expression of PDGFR-B in mesenchymal progenitor cells, pericytes, and smooth muscle cells is required for mural cell proliferation, migration, and incorporation in vessel walls.

Soriano (1994) demonstrated that PDGFR-B gene knockout impaired kidney development in mice because of glomerular development irregularities secondary to an absence of mesangial cells, structurally and functionally related to pericytes. In addition, hematologic abnormalities, such as anemia, thrombocytopenia, erythroblastosis, and arterial dilation, were also present.

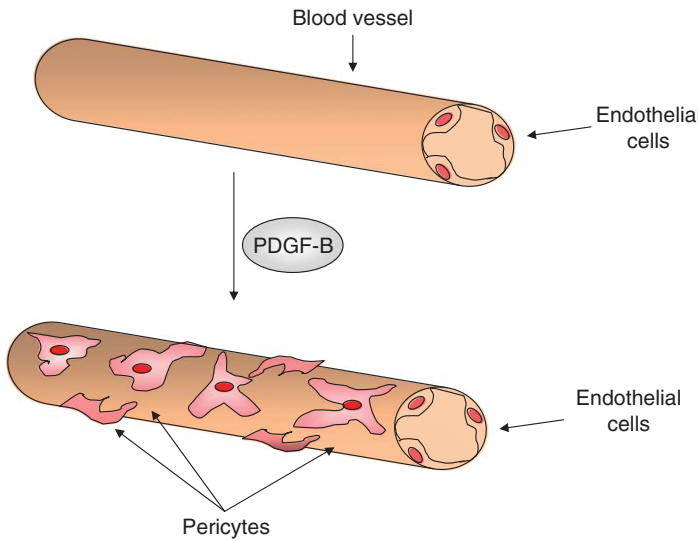

Figure 1.7 Schematic representation of pericyte recruitment to a newly formed vessel induced by PDGF-B. PDGF-B released from endothelial cells acts as a chemoattractant by stimulating PDGFR-B on pericytes, which causes their recruitment.

Mice mutated in PDGF-B displayed a similar phenotype, died perinatally as a result of hemorrhage, and presented failure of recruitment of pericytes (Lindahl *et al.*, 1997, 1998). A detailed analysis of vessel development in both PDGF-B and PDGFR-B mutant embryos showed that smooth muscle cells and pericytes initially formed around vessels but, as vessels sprouted and enlarged, PDGF signaling was required for comigration and proliferation of supporting cells (Hellstrom *et al.*, 1999). Lymphatics acquired ectopic smooth muscle cells in PDGFR-B knockout mice (Foo *et al.*, 2006).

PDGFR-B function may involve cooperation with a family of G protein-coupled receptors (S1P1–5) that bind to sphingosine 1-phosphate, a sphingolipid secreted by endothelial cells (Spiegel and Milstein, 2003). Gene-targeting experiments indicated that S1P1 (also known as EDG1), S1P2, and S1P3 have important roles during vascular morphogenesis and that the numbers of pericytes and smooth muscle cells are compromised in S1P1-deficient mice (Liu *et al.*, 2000; Kono *et al.*, 2004). The similarity between phenotypes of PDGF-B/PDGFR-B and S1P1 knockout mice indicates that signaling through the EDG1 receptor, which is expressed by mural cells, is another key pathway for mural cell recruitment (Kluk and Hla, 2002).

2.3. Angiopoietins, Tie-1, and Tie-2

The angiopoietin (Ang) family comprises at least four secreted proteins, Ang-1, -2, -3, and -4, all of which bind to the endothelial-specific receptor tyrosine kinase Tie-2, whereas Tie-1 is an orphan receptor tyrosine kinase. It is well documented that Angs play a critical role in endothelial sprouting, vessel wall remodeling, and mural cell recruitment (Thurston, 2003) (Fig. 1.8).

Vasculogenesis proceeds normally in embryos lacking both Tie-1 and Tie-2, although they died early as a result of multiple cardiovascular defects (Puri *et al.*, 1999; Suri *et al.*, 1996). In the absence of Tie-1 the first symptom is the development of edema, after which there are multiple hemorrhages. Because there are no reported changes in the vascular patterning in the mutant mice, it has been suggested that Tie-1 plays an important role late in development in establishing/maintaining the integrity of an existing vascular network. Mosaic analyses have shown that endothelial cells lacking Tie-1 contribute to the embryonic vasculature on day 10.5 as efficiently as wild-type cells. From day 15, Tie-1 mutant cells are selected against in the capillary plexuses of the brain and kidney. Thus, Tie-1 seems to play a significant role in late phases of organ-specific angiogenesis (Puri *et al.*, 1999).

Mutation of Tie-2 did not affect initial formation of blood vessels, but embryos died in midgestation with major defects in vascular remodeling and stability (Sato *et al.*, 1995). In the yolk sac and aorta, the number of endothelial cells appeared to be reduced and the surviving cells

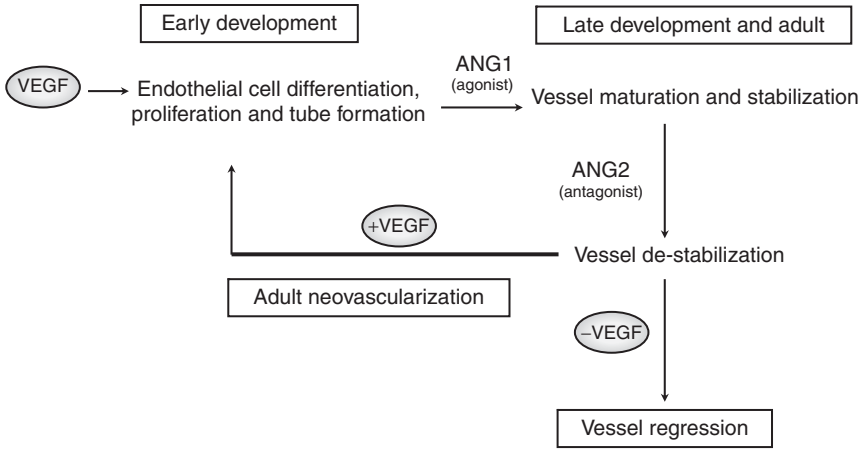

Figure 1.8 Schematic representation of the effects of VEGF, Ang-1, and Ang-2 on vascular structure.

looked disorganized. There were blood cells in the yolk sac cavity as well as in the trunk of the embryo, suggesting that the vessels may have ruptured. The heart appeared to be poorly developed, with a sparsely populated endocardium and an immature myocardium. Blood vessels were enlarged, with fewer branches, and endothelial cells tended to round up, dissociate from the underlying support cells and extracellular matrix, and undergo apoptosis. Chimera studies showed that the role of Tie-2 was specific to the late stages of capillary formation (Partanen *et al.*, 1996). Mosaic analysis of Tie-2-deficient and normal embryos revealed an absolute requirement for Tie-2 in the endocardium, whereas Tie-2 and Tie-1 double-mutant cells were dispensable for the initial formation of vasculature, but required for the microvasculature during late organogenesis and essential for the blood vessel of the adult (Puri *et al.*, 1999).

Ang-2 can bind to the Tie-2 receptor but does not activate it; rather, it seems to act as an antagonist, counteracting the effects of Ang-1. Consistent with this, overexpression of Ang-2 in embryos resulted in embryonic death on day 9.5 because of defects resembling those of knockouts of Ang-1 or Tie-2 (Maisonpierre *et al.*, 1997). The embryos showed massive vascular disruptions, which, as when Ang-1 was absent, appeared to be caused by changes in both endothelial and smooth muscle cell components. The Ang-2-overexpressing mutant embryos had a more severe phenotype compared with mutant embryos lacking Ang-1.

Knockout embryos lacking Ang-1 displayed failure of endothelial cell adherence and interaction with perivascular cells and extracellular matrix (Davis and Yancopoulos, 1999). Mice lacking Ang-1 had a phenotype

similar to that seen in the absence of Tie-2 (Suri *et al.*, 1996). The mutant embryos died around day 11.5 with cardiovascular abnormalities. Heart development was severely retarded such that by day 11.5 the ventricles were small, the atria were almost invisible, and the coronary artery bed was significantly sparse. Histologic analysis revealed the endocardial layer to have separated from the underlying myocardial layer as well as an impressive reduction in myocardial trabecular formation. Vascular beds at all other sites of Ang-1 expression were also adversely affected. In the yolk sac and forebrain, the vasculature resembled an immature capillary plexus wherein the vessels were dilated and uniformly sized without division into larger and smaller vessels. Tissue factor knockout mice displayed defects similar to those of Ang-$1^{-/-}$ mice in terms of pericyte recruitment in the yolk sac vasculature (Carmeliet *et al.*, 1996b). Absence of tissue factor leads to embryonic lethality between days 9.5 and 10.5. The phenotype of this mouse includes an abnormal yolk sac vasculature characterized by a lack of smooth muscle cells.

In the absence of mural cells, recombinant Ang-1 restored a hierarchical order of the larger vessels, and rescued edema and hemorrhage in the growing retinal vasculature of mouse neonates (Uemura *et al.*, 2002). Unlike mouse embryos lacking VEGF or VEGFR-2, embryos lacking Ang-1 or Tie-2 develop an essentially normal primary vasculature. However, this vasculature fails to undergo further remodeling. The most prominent defects are in the heart, with problems in the association between the endocardium and the underlying myocardium as well as in trabecular formation, and also in the remodeling of many vascular beds into large and small vessels. In these vascular beds, as in the heart, ultrastructural analysis indicated that endothelial cells failed to associate appropriately with the underlying support cells (Suri *et al.*, 1996). On the basis of endothelium-restricted expression of Tie-2 and the dominant smooth muscle cell expression of Angs, it appears that recruitment of smooth muscle cells or pericytes into the proximity of endothelial cells of newly formed vessels is required for Tie-2 activation.

The patterning of the lymphatic network and smooth muscle cell recruitment to the collecting lymphatics are defective in Ang-2-deficient mice.

Transgenic overexpression of Ang-1 in skin resulted in pronounced hypervascularization with the production of many compact stable vessels resistant to leakage (Suri *et al.*, 1998; Thurston *et al.*, 1999). When Ang-1 was made available at high levels in the skin, the resultant mice were so highly vascularized that the skin appeared red. The most marked increase was in vessel size. This contrasted with the effects of VEGF overexpression, which also led to hypervascularization but with the formation of large, leaky, simple endothelial tubes, unprotected by supporting cells (Drake and Little, 1995).

2.4. Hypoxia-inducible factor 1

Many genes involved in angiogenesis, including the VEGF gene itself, are up-regulated by hypoxia in both normal and pathological conditions (Semenza, 1999). Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that is selectively stabilized and activated under hypoxic conditions, and that coordinates the adaptive response of tissues to hypoxia (Semenza, 1999). Functionally, HIF-1 exists as a β heterodimer, the activation of which is dependent on stabilization of the oxygen-sensitive degradation domain of the α subunit by the ubiquitin-proteasome pathway (Semenza, 1999).

Gene-targeting experiments have clearly demonstrated that HIF-1α is required for embryonic vascularization, as major defects in early vascular development were observed in homozygous mice lacking HIF-1α (Iyer *et al.*, 1998; Ryan *et al.*, 1998). Targeted mutation of HIF-1α in mice led to a series of malformations of the embryo, including abnormal neural folding, myocardial hyperplasia, and defects in both embryonic and extraembryonic vasculature. Sections of HIF-$1\alpha^{-/-}$ embryos showed massively enlarged neural vessels, with dilated and disorganized vessels (Iyer *et al.*, 1998; Ryan *et al.*, 1998). These defects could not be explained by reduced expression of VEGF, because VEGF was found upregulated even in HIF-1α null embryos (Kotch *et al.*, 1999). Thus, vascular regression was the result of cell death rather than VEGF deficiency.

2.5. Other factors

2.5.1. Neuropilins

The neuropilins (NRPs) are a small family of transmembrane proteins. NRPs bind certain members of the VEGF family: NRP-1 and NRP-2 bind $VEGF_{165}$ and PlGF; NRP-1 also binds VEGF-B; NRP-2 also binds $VEGF_{145}$ and VEGF-C (Karkkainen *et al.*, 2001; Neufeld *et al.*, 2002). NRP-1 and NRP-2 are mediators of neuronal guidance and angiogenesis. NRP-1 is found in arteries, whereas NRP-2 is restricted to veins and lymphatic vessels (Gu *et al.*, 2003; Stalmans *et al.*, 2002; Yuan *et al.*, 2002).

NRP-1-deficient embryos died between days 12.5 and 13.5 and presented cardiovascular failure (Kitsukawa *et al.*, 1997), whereas chimeric mice overexpressing NRP-1 showed excess capillaries and blood vessels, dilatation of vessels, and malformed hearts (Kitsukawa *et al.*, 1995). In yolk sac and embryos of mice lacking a functional NRP-1 gene the vascular network of large and small vessels was disorganized, the capillary networks were sparse, and normal branching did not occur. In the central nervous system, capillary invasion was delayed and the capillary networks were disorganized and had degenerated. Finally, the mutant embryos showed

abnormal heart development, and the development of heart outflow tracts was disturbed (Kawasaki *et al.*, 1999). Defects in vascular patterning and filopodial extension in NRP-1 knockout mice were relatively mild, which indicates that there are important roles for other VEGFRs in the tip cell guidance process (Gerhardt *et al.*, 2004). Vessel branching defects in NRP-1-deficient mice point to a role in tip cell guidance, but mouse mutants lacking the NRP-1-binding VEGF isoform showed distinct vessel patterning defects (Ruhrberg *et al.*, 2002), suggesting a more selective requirement for NRP-1 during vessel branching.

NRP-2, which is expressed in lymphatic vessels, can interact with VEGF-C and VEGF-D and is essential for lymphangiogenesis (Karpanen *et al.*, 2006; Yuan *et al.*, 2002). NRP-2 knockout mice showed reduced lymphatic endothelial cell proliferation and failed to develop small-diameter lymphatic vessels, whereas the vasculature developed almost normally (Yuan *et al.*, 2002). Nevertheless, NRP-2 plays an important role in vasculogenesis, because mice lacking both NRPs displayed vascular abnormalities that were much more severe than the abnormalities seen in mice lacking a functional NRP-1 gene (Takashima *et al.*, 2002).

Combined knockouts for NRP-1 and NRP-2 led to vasculogenesis defects and failure to assemble the primary vascular plexus (Takashima *et al.*, 2002). Transgenic mice, in which both NRP-1 and NRP-2 were targeted, had a more severe abnormal vascular phenotype than either NRP-1 or NRP-2 single knockout mice, died *in utero* on day 8.5, and their yolk sacs were avascular. Mice that were homozygous for one gene but heterozygous for the other were also embryonic lethal, surviving to day 10 to 10.5, and their vascular phenotypes were abnormal. The yolk sacs, although of normal size, displayed the absence of a capillary bed and the presence of large avascular spaces between the blood vessels. The embryos displayed blood vessels that were heterogeneous in size, large avascular regions in the head and trunk, and unconnected blood vessel sprouts, and had multiple hemorrhages.

2.5.2. Notch and Delta

Notch signaling is a highly conserved pathway, initially discovered in *Drosophila* development (Baron *et al.*, 2002). Although the contribution of Notch in cell fate determination of neuronal, hematopoietic, and muscle cells has been acknowledged, its impact on the cardiovascular system was recognized only more recently.

There are four Notch receptor (Notch 1–4) and five ligands (Jagged-1 and -2, and Delta-1, -3, and -4) (Iso *et al.*, 2003). All the receptors and ligands have been expressed in at least one vascular compartment, for example, arteries, veins, capillaries, vascular smooth muscle cells, or pericytes. In mouse embryos, Notch-1, -3, and -4, Delta-4, and Jagged-1 and -2 are all expressed in association with the vasculature by days 10 to 17

(Villa *et al.*, 2001). Of these, Notch-1 and -4, Delta-4, and Jagged-1 and -2 were specifically expressed in the arterial endothelium and were found at low levels in veins. Notch-3 was localized to the smooth muscle cells surrounding arteries but not veins, whereas Jagged-1 was expressed in both endothelial and smooth muscle cells associated with arterial vessels. Expression of Notch-4 and Delta-4 appears to be entirely restricted to the endothelium, including the developing capillaries (Uyttendaele *et al.*, 1996). The knockout phenotypes and their defective arteriovenous marker expression support important roles for these molecules in vascular differentiation (Duarte *et al.*, 2004; Gale *et al.*, 2004; Krebs *et al.*, 2000). Mouse embryos deficient for Jagged-1, Notch-1, and Notch-1/Notch-4 died between days 9.5 and 10.5 and displayed severely disorganized vasculature (Krebs *et al.*, 2000). Mice deficient for the Notch-1 gene showed abnormalities that affected somites and the heart and blood vessels. Mutant embryos and yolk sac were able to form the primary vascular plexus, indicating that Notch function is not necessary for vasculogenesis. However, the yolk sac of Notch-deficient mice did not remodel the primitive plexus into the typical branching architecture of larger to smaller vessels. Furthermore, in these mutant embryos, the dorsal aortas and cardinal vein formed, but were smaller than wild-type vessels and appeared collapsed (Krebs *et al.*, 2000). Gene inactivation has also been performed for Notch-2, -3, and -4. Alone, none of these mutants mimicked the effects demonstrated in Notch-1 mutant embryos. Combination mutants have been created for Notch-1 and -4. Mice deficient in Notch-4 were normal. However, 50% of mice deficient in Notch-1 and -4 had a more severe vascular phenotype than did Notch-1 knockout mice, suggesting that Notch-1 and -4 have partially redundant roles (Krebs *et al.*, 2000).

Mice with defects in genes encoding Notch, Notch ligands, and components of the Notch signaling cascade displayed vascular defects, such as Alagille's syndrome, a developmental disorder with vascular defects and CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy), an adult-onset vascular dementia in which affected persons are at increased risk of stroke and coronary occlusion (Shawber and Kitajewski, 2004). In the majority of patients CADASIL has been found to correlate with missense mutation in Notch-3. CADASIL patients exhibit a degeneration of the smooth muscle layer surrounding the cerebral arteries and skin arterioles. This human syndrome clearly demonstrates a requirement for Notch signaling in vascular development and adult vessel maintenance.

Notch receptors and their Delta-like-4 (Dll-4) ligand are essential for sprouting during mouse development. Reduced levels of Dll-4 or blocking of Notch signaling enhances the formation of tip cells, resulting in dramatic increases sprouting branching and fusion of endothelial tubes (Hellstrom *et al.*, 2007; Lobov *et al.*, 2007; Sainson *et al.*, 2005; Suchting *et al.*, 2007).

Dll-4 expression is induced in the tip cell, whereas the activation of Notch signaling in neighboring endothelial cells is though to suppress sprouting of these cells (Hellstrom *et al.*, 2007; Suchting *et al.*, 2007).

2.5.3. Cadherins

Endothelial cells express both neural (N)-cadherin (Liaw *et al.*, 1990; Salomon *et al.*, 1992) and vascular endothelial (VE)-cadherin (Lampugnani *et al.*, 1992). Despite similar expression levels, only VE-cadherin is expressed at cell–cell junctions, whereas N-cadherin is spread over the cell membrane, In fact, whereas VE-cadherin mediates homotypic interactions resulting in endothelial cell–cell attachment, N-cadherin seems essentially to participate in the anchorage of other N-cadherin-expressing cells, such as pericytes, to the endothelium.

Targeted deletion of these genes in mice led to early embryonic death with associated severe vascular anomalies (Carmeliet *et al.*, 1999a; Gory-Faure *et al.*, 1999; Radice *et al.*, 1997). VE-cadherin homozygous null embryonic stem (ES) cells showed disorganized vessel formation, but expressed normal levels of other endothelial cell markers (Vittet *et al.*, 1997). These authors used embryoid body formation from VE-cadherin$^{-/-}$ ES cells and demonstrated that whereas endothelial cells could differentiate from the mesoderm, they could not organize unto a definitive vascular plexus. VE-cadherin$^{-/-}$ embryos did not demonstrate defective cell–cell interactions, suggesting that VE-cadherin is dispensable for endothelial homophilic interaction but essential for some additional aspects of angiogenic remodeling.

Targeted inactivation or truncation of the β-catenin-binding cytosolic domain of the VE-cadherin gene did not affect assembly of endothelial cells in vascular plexi, but impaired their subsequent remodeling and maturation, causing lethality on day 9.5 of gestation (Carmeliet *et al.*, 1999b). Moreover, deficiency or truncation of VE-cadherin induced endothelial apoptosis and abolished transmission of the endothelial survival signal by VEGF-A to Akt kinase and Bcl-2 via reduced complex formation with VEGFR-2, β-catenin, and phosphoinositide-3-kinase (Carmeliet *et al.*, 1999b). In VE-cadherin knockouts, Carmeliet *et al.* (1999b) concluded that because lumenized vessels were evident, the basis of the defects was a failure in the process of angiogenesis. By contrast, Gory-Faure *et al.* (1999), noting defects in the yolk sac vessels concomitant with apparent normal intraembryonic vessels (i.e., dorsal aortas), concluded that extraembryonic vasculogenesis was dependent on VE-cadherin activity, whereas intraembryonic vasculogenesis was not. Crosby *et al.* (2005) investigated VE-cadherin in blood vessel morphogenesis and established a temporal correlation linking failed vessel morphogenesis in VE-cadherin null embryos to a specific step in vasculogenesis. They concluded that the events of *de novo* blood vessel formation up to the point at which vascular epithelium forms are not dependent on VE-cadherin

and that VE-cadherin, the expression of which is up-regulated after vascular epithelialization, is required to prevent the disassembly of nascent blood vessels.

Mice lacking N-cadherin showed a defect in yolk sac vascularization (Radice *et al.*, 1997), with *in utero* death on day 9.5. The major abnormality detected in N-cadherin null mutant mice was dramatic alteration in the developing heart and myocardium; the yolk sac defects could be merely a reflection of altered cardiac performance.

2.5.4. Ephrins

Differentiation of arteries and veins was thought to be governed by hemodynamic forces, molding vessels from the primary vascular plexus. Murray (1926) postulated that vessels adapt to flow in order to optimize the shear stress to which they are subjected. These studies have shown that flow can alter lumenal dimensions of arterial segments. However, labeling experiments done in zebrafish indicated that the arterial and venous fates of endothelial precursors may be determined before formation of the blood vessels (Zhang *et al.*, 2001). These authors monitored individual angioblasts and found that, contrary to expectations, all the progeny of a single angioblast formed either veins or arteries, never both. In other words, each angioblast was already specified as to whether it would form aorta or cardinal vein.

It is now clear that arteries and veins can be distinguished from each other at the molecular level and that distinct signaling pathways operate to grow arteries and veins preferentially.

The ephrins and their Eph receptors (the Eph family of receptor tyrosine kinases comprises 15 members that are activated by the ephrins) are a novel class of proteins essential for the proper development of new blood vessels and have begun to serve as the first reliable marker capable of distinguishing arterial from venous vessels. The discovery that members of the ephrin family are differentially expressed in arteries and veins from early stages of development, before development of a functional circulation, was one of the first indications that artery–vein identity is intrinsically programmed. Eph-B2 marks arterial endothelial cells and smooth muscle cells, whereas Eph-B4, a receptor for Eph-B2, marks only veins. Moreover, Eph-B2–Eph-B4 signaling participates in the formation of arteriovenous anastomoses by arresting VEGF- and Ang-1-induced endothelial cell proliferation/migration at the arteriovenous interface (Gerety *et al.*, 1999; Wang *et al.*, 1998; Zhang *et al.*, 2001).

Mutations of Eph-B2 and Eph-B4 both lead to early embryonic lethality around days 9.5–10.5 (Adams *et al.*, 1999; Gerety and Anderson, 2002; Gerety *et al.*, 1999; Wang *et al.*, 1998). Mouse embryos lacking Eph-B2 and Eph-B4 suffer fatal defects in early angiogenic remodeling that are somewhat reminiscent of those seen in mice lacking Ang-1 or Tie-2.

Vasculogenesis is halted at the primary plexus stage, endothelial cell are disorganized, and many features of angiogenic remodeling are absent (Adams and Klein, 2000). The observation that Eph-B2-deficient embryos showed defective vascularization of the nervous system, which normally occurs by angiogenic sprouting from adjacent vessels, suggests a role for Eph-B2 as an inducer of capillary sprouting *in vivo* (Adams *et al.*, 1999). Although ephrin/Eph signaling appears not to be essential for the initial specification of endothelial cell fates, interactions between Eph-B2 and Eph-B4 at the arteriovenous interface are required to provide repulsion signals for the maintenance of boundaries between these vessels.

Smooth muscle cells are found on terminal lymphatics in mice that lack Eph-B2 in mural cells. In these mutants, pericytes and smooth muscle cells fail to associate stably with blood vessels and some migrate to lymphatics (Foo *et al.*, 2006).

2.5.5. Chicken ovalbumin upstream promoter transcription factor II

Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), a member of the orphan nuclear receptor superfamily, is expressed in venous endothelium. In mice that lack COUP-TFII, veins acquire arterial characteristics and express components of the Notch pathway and Eph-B2 (You *et al.*, 2005).

2.5.6. Fibronectin

Vasculogenesis takes place in a fibronectin-rich extracellular matrix (Risau and Lemmon, 1988). As soon the basic vascular network is established, fibronectin decreases in the vicinity of developing blood vessels and endothelial cells begin to produce laminin and collagen type IV in increasing amounts (Ausprunk *et al.*, 1991; Drake *et al.*, 1990; Risau and Lemmon, 1988).

Fibronectin null nice died *in utero* by days 10 to 11 and exhibited severe defects in blood vessel and heart development (George *et al.*, 1993). Fibronectin promotes VEGF-induced differentiation of peripheral blood-derived endothelial progenitors to endothelial cells (Wijelath *et al.*, 2004).

2.5.7. Netrins

Netrins are a family of secreted matrix-binding proteins with homology to laminin (Serafini *et al.*, 1994). Three members of the netrin gene family have been identified in mammals: netrin-1, netrin-3, and β-netrin/netrin-4. Loss of function in mice or fish of UNC5B, a receptor for netrins, which is strongly expressed in capillaries and endothelial tip cells, led to increased endothelial sprouting, whereas stimulation of endothelial cells with the ligand netrin-1 led to retraction of tip cell filopodia, which is consistent with negative regulation of blood vessel growth by netrins and UNC5B, suggesting that netrins may act as attractants or repellents in both the

nervous and vascular systems (Lu *et al.*, 2004). Vessel branching was selectively affected in UNC5B-deficient mice as neither changes in arteriovenous marker expression, nor in vessel wall assembly or endothelial cell proliferation and apoptosis, could be detected.

2.5.8. Integrins

Integrins are heterodimeric transmembrane molecules expressed by almost all cells except erythrocytes. At least five integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$) are expressed at some point on endothelial cells or pericytes.

In a study of the α_5 integrin, Yang *et al.* (1993) deleted the α_5 gene by homologous recombination. The homozygous null mutations were embryonically lethal by days 10 to 11 and although mice developed a heart and vascular system, they showed defects in both the extraembryonic and embryonic vascular beds.

α_v null mutant mice revealed extensive angiogenesis and vasculogenesis before embryonic death (Hynes *et al.*, 1999): 80% of the embryos died *in utero*, most likely as a result of placental insufficiency, and 20% were born alive, but developed intestinal and intracerebral hemorrhages. Similarly, β_3 null mice showed normal vascular development with some placental insufficiency and thromboasthenia at birth (Hodivala-Dilke *et al.*, 1999).

2.5.9. Hedgehog

Hedgehog (Hh) is a secreted signaling molecule that serves multiple roles during embryonic development. There are three Hh genes in the mouse: sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh) (McMahon *et al.*, 2003). Ihh mutant yolk sacs were able to form endothelial cell-containing blood islands and a primary capillary plexus, and even displayed a limited ability to remodel the vessels. The vessels formed in the absence of Ihh signaling were smaller, less organized, and appeared flattened or collapsed (Byrd *et al.*, 2002). A compromised yolk sac vasculature likely explains why 50% of Ihh-deficient embryos died at midgestation (St Jacques *et al.*, 1999).

2.5.10. Semaphorins

Semaphorins (Semas) comprise a large family of phylogenetically conserved secreted and membrane-bound proteins. More than 20 Semas are known to date. Eight Sema subclasses are distinguished on the basis of structural features and sequence similarities: subclasses 1 and 2 comprise invertebrate Semas, subclasses 3–7 represent vertebrate Semas, and subclass V comprises viral Semas (Pasterkamp and Kolodkin, 2003). The role of Semas in vascular development has been analyzed in several models, and they have been shown to be regulators of vascular development.

The class 3 Semas (e.g., Sema 3E), which are the best characterized Semas, control both axon guidance and vascular patterning. Semas mediate their effects by interacting with receptor complexes that are formed by (semaphorin-binding) NRP and (signal-transducing) plexin family transmembrane proteins (Giger *et al.*, 2000; Kruger *et al.*, 2005). Gu *et al.* (2003) performed genetic manipulation in mice to selectively disrupt NRP-1 interactions with Sema-3, while retaining VEGF binding. Neural development was severely affected in these mice, whereas overall vascular development was normal, indicating that Sema-3/NRP signaling is dispensable for vascular development. Sema 3E can signal through plexin D1 independently of NRP (Gu *et al.*, 2005). Loss of plexin D1 caused exuberant sprouting of intersomitic vessels into Sema 3E-expressing somitic tissue and loss of the normal segmental blood vessel pattern, which suggests that Semas are repulsive for endothelial cells (Gitler *et al.*, 2004).

2.5.11. Vascular cell adhesion molecule-1

Using targeted disruption of the vascular cell adhesion molecule-1 (VCAM-1) gene, Kwee *et al.* (1995) and Gutner *et al.* (1995) demonstrated that a null mutation resulted in a failure of placental development and this resulted in death around embryonic days 9.5–10.

2.5.12. Prospero-related homeobox-1 transcription factor

Prospero-related homeobox (PROX)-1 function is essential for lymphatic development. PROX-1-positive endothelial cells up-regulate a number of lymphatic endothelial-specific markers, bud from the cardinal veins, and migrate to form the primary lymph sac, from which lymphatic capillaries sprout to form the primary lymphatic plexus. Inactivation of PROX-1 in mice resulted in defective extension of endothelial cells from the cardinal vein, loss of lymphatic marker expression, and lack of the lymphatic vasculature (Wigle and Oliver, 1999).

3. Concluding Remarks

The development of efficient transgenic technologies in mice has allowed the study of the consequences of genetic alterations on angiogenesis and lymphangiogenesis. Mouse studies have been invaluable to the identification of key components in the molecular regulation of endothelial differentiation. A full extrapolation of experimental data from genetically modified mice to humans must be made with caution. In fact, mice differ from humans in many ways, necessitating careful and in-depth analysis of these mouse models.

The purpose of gene knockout is to ablate the function of a targeted gene in order to discern its role *in vivo*. However, the fact that a specific mutation has been present in the mouse from the time of its conception may lead to false conclusions, because of the inability to distinguish between phenotypic changes due to the mutation itself and to changes caused by adaptation and compensation for the mutation.

Continued progress in the development of conditional knockout technologies combined with the further characterization of early vascular lineage-specific promoters will provide invaluable tools for investigations into specific gene function and expression, as well as vascular lineage analysis.

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CHAPTER TWO

Morphogenesis in Giant-Celled Algae

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Contents

Abstract

The giant-celled algae, which consist of cells reaching millimeters in size, some even centimeters, exhibit unique cell architecture and physiological characteristics. Their cells display a variety of morphogenetic phenomena, that is, growth, division, differentiation, and reproductive cell formation, as well as wound-healing responses. Studies using immunofluorescence microscopy and pharmacological approaches have shown that microtubules and/or actin filaments are involved in many of these events through the generation of intracellular movement of cell components or entire protoplasmic contents and the spatial control of cell activities in specific areas of the giant cells. A number of environmental factors including physical stimuli, such as light and gravity, invoke localized but also generalized cellular reactions. These have been extensively investigated to understand the regulation of morphogenesis, in particular addressing cytoskeletal and endomembrane dynamics, electrophysiological

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elements affecting ion fluxes, and the synthesis and mechanical properties of the cell wall. Some of the regulatory pathways involve signal transduction and hormonal control, as in other organisms. The giant unicellular green alga *Acetabularia*, which has proven its usefulness as an experimental model in early amputation/grafting experiments, will potentially once again serve as a useful model organism for studying the role of gene expression in orchestrating cellular morphogenesis.

Key Words: Cell wall, Cell division, Cell growth, Cell motility, Coenocyte, Cytoskeleton, Posttranscriptional control, Wound healing.

1. Introduction

Algae are a polyphyletic group of photosynthetic organisms usually living in aquatic environments (Bold and Wynne, 1895; Graham and Wilcox, 2000). Some species of macroscopic algae consist of cells more than a millimeter in size. These algae are often coenocytic, that is to say, a giant mass of protoplasm containing a huge number of nuclei occupies the macroscopic, often siphonous thallus. Communication and material exchange between remote regions of the thallus is ensured by long-distance transport of organelles uninterrupted by any cross wall. Some members in this group of algae, however, remain uninuclear throughout much of their life cycles and still acquire giant dimensions. Because such giant-celled algae are visible to the naked eye, researchers have been able to observe and manipulate these cells more easily than cells in other organisms. Therefore, giant-celled algae have been used for various studies in cell biological research for many years (Bonotto and Berger, 1994). Similar to other organisms, the cells in giant-celled algae also exhibit a variety of morphogenetic events such as growth, branching, cell division, and wound-healing reactions. Again, many of these events are also readily visible under a dissecting microscope. Because of the large size of the cells it is possible to study the localization of material(s), event(s), and function(s) within a cell in relation to the morphogenetic event occurring at a specific locality of the cell. Therefore, there have been a number of excellent studies concerning the morphogenesis in the giant-celled algae. In this review, we outline the biological features of giant-celled algae, describe the morphogenesis that occurs in their cells, and review some selected examples of such studies relating each morphogenetic event with cytoskeletons and other cellular factors, in order to project possible directions of future research. The genera we cover in this review include *Acetabularia*, *Acrosiphonia*, *Boodlea*, *Bryopsis*, *Chaetomorpha*, *Chamaedoris*, *Chara/Nitella*, *Caulerpa*, *Derbesia/Halicystis*, *Dictyosphaeria*, *Ernodesmis*, *Griffithsia*, *Trichosolen*, *Valonia*, *Vaucheria*, and *Ventricaria*.

Other giant-celled algae (e.g., *Udotea*, *Halimeda*, and *Bornetella*) are excluded because they have complex and/or multiaxial thallus structures.

2. Biological Features of Giant-Celled Algae

2.1. Taxonomic and ecological aspects

According to the taxonomic classification by Graham and Wilcox (2000), giant-celled algae occur in five algal classes: Chlorophyceae, Charophyceae, Ulvophyceae, Rhodophyceae, and Xanthophyceae. The Ulvophyceae contain a variety of taxa of giant-celled algae, and consequently as many as 13 genera in 4 orders are dealt with in this review. In contrast, with respect to cell biological studies, the number of taxa of giant-celled algae is quite limited in other classes. *Hydrodictyon* is the only genus with truly giant cells in the Chlorophyceae. Other large-celled examples such as *Characiosiphon* and *Eremosphaera* (Chlorococcales) are not treated here. For a comparison of morphogenetic mechanisms between *Micrasterias* (Desmidiales) and *Acetabularia* (Dasycladales) see Lütz-Meindl and Menzel (2000). The Charophyceae contain giant-celled genera such as *Chara* and *Nitella*, and related taxa belonging to the order Charales. The Vaucheriaceae, which contain *Vaucheria* and *Botrydium*, is the only family in the Xanthophyceae having giant-celled taxa, and in fact, no other class in the Chromophyta features giant cells. *Griffithsia*, a member of the order Ceramiales, is the only genus with giant-celled species in the Rhodophyta. Many other genera in this class contain multinuclear cells; however, none of them is giant-celled, except for *Griffithsia*.

The salinity seems to be an important ecophysiological condition to which the cells of aquatic organisms are adapted. Giant-celled algae live in a variety of saline environments, marine, brackish, and fresh water habitats. Most species in the Ulvophyceae as well as the red alga *Griffithsia* live in marine environments. In contrast, all characean algae are fresh to brackish water algae. Such broad ranges of salinity in the environments for the giant-celled algae indicate, that they have acquired distinct mechanisms for maintaining intra/extracellular osmotic conditions and for regulating their turgor pressure (Bisson, 1995; Bisson *et al.*, 2006).

2.2. Cellular organization

2.2.1. Thallus morphologies

Giant-celled algae evoke confusion in the discrimination between unicellular and multicellular organisms because they are often "unicellular," when they are growing into vegetative macroscopic individuals, as in the case of *Acetabularia*, *Bryopsis*, *Caulerpa*, *Trichosolen*, *Vaucheria*, and *Ventricaria*. Other species of the giant-celled algae are multicellular in early developmental

stages of individuals. For example, *Acrosiphonia*, *Griffithsia*, and *Chaetomorpha* are such multicellular giant-celled algae. Here, we distinguish unicellular from multicellular organisms by the number of cells that constitute a single individual, when they complete their vegetative growth in their life history. According to such a criterion, we classify some species of giant-celled algae as multicellular giant-celled algae because they multiply their cell number from a single cell to multiple cells before reproductive maturity, such as *Dictyosphaeria* and *Siphonocladus*.

External morphology varies among species of giant-celled algae except for being macroscopic in size. There are many filamentous species, some containing branches, such as *Acrosiphonia*, *Bryopsis*, *Caulerpa*, *Trichosolen*, *Griffithsia*, and *Vaucheria*. Others are not branched, for example, *Acetabularia* and *Chaetomorpha*. In addition to filamentous species, many siphonocladalean algae have globular to oblong forms such as *Boergesenia*, *Dictyosphaeria*, *Valonia*, and *Ventricaria*.

From the observations of thalli in both natural environment and laboratory culture, vegetative growth of individuals in the giant-celled algae, such as *Acetabularia*, *Boergesenia*, *Dictyosphaeria*, *Valonia*, and *Ventricaria* is clearly defined. Growth terminates at a stage, when the thallus reaches a certain size, starts with cell division, or begins with the formation of reproductive cells. Other unicellular algae, for example, *Caulerpa* and *Vaucheria*, and multicellular algae such as *Acrosiphonia*, *Chara*, and *Griffithsia*, exhibit indefinite growth (growth patterns repeat over and over); their vegetative growth continues as long as environmental conditions allow, in the natural environment or in culture vessels. In fact, *Caulerpa* can grow to be several feet and species such as *Caulerpa cactoides* are as thick as a finger, and, for some, it is the ''largest unicellular organism on earth'' (Jacobs, 1994). More precisely, it is a coenocyte and can be regarded as the organism with the largest volume of unpartitioned protoplasm. *Bryopsis* is unique in the mode of its vegetative growth. The gametophyte of *Bryopsis plumosa* is formed by definite growth. It consists of a thallus of several centimeters in length with an axis and pinnate side branches. Some of the side branches are plugged off from the main axis at maturity and transform into gametangia. However, other sterile side branches often grow into new axes bearing secondary side branches similar to those in the original thallus. Moreover, cell fragments excised from the axis or side branches may also grow into complete pinnate gametophytes (Mine *et al.*, 1996). Thus in *B. plumosa* the growth of the axis is definite, whereas that of the side branches is indefinite.

2.2.2. Intracellular organization

It is a common feature of the intracellular structure in giant-celled algae that the cytoplasm is usually restricted to a thin layer in the cell periphery and the remaining cell volume is occupied by a huge central vacuole. In some giant-celled algae, for example, *Bryopsis*, *Caulerpa*, *Chara*, and *Valonia*, the

peripheral cytoplasmic layer is divided into sublayers, each of which contains certain type(s) of intracellular structures, such as nuclei, chloroplasts, cytoskeletal elements, and other organelles (McNaughton and Goff, 1990; Menzel and Elsner-Menzel, 1989c; Menzel and Schliwa, 1986a; Shihira-Ishikawa, 1987; Williamson, 1992). In the other algae such as *Acetabularia* and *Vaucheria*, such sublayers of organelles are not as obvious (Menzel, 1994; Takahashi *et al.*, 2001). Despite the fact that the protoplasm is limited to only a thin layer along the cell periphery, the total volume of the protoplasm per cell in these algae is much larger than that in other organisms consisting of smaller cells. Yet, the relationships between genome size and cytoplasmic volume (Goff and Coleman, 1987) seems to be maintained in the giant-celled algae, because most are multinucleate coenocytes. Others such as *Acetabularia* (Berger *et al.*, 1987; Menzel, 1994) and *Bryopsis* (Neumann, 1969), which are uninucleate diploids during the initial vegetative state, feature huge primary nuclei in the rhizoidal area. These giant nuclei contain several conspicuous, sausage-like nucleoli, which at least in the case of *Acetabularia* contain amplified extrachromosomal copies of the ribosomal RNA cistrons (Berger and Schweiger, 1975), and the same is probably true for the other members of the Dasycladales (Berger *et al.*, 1987; Menzel, 1994).

Nuclei in the multinuclear cells may be arranged randomly as in *Bryopsis* and *Derbesia* (McNaughton and Goff, 1990; Okuda *et al.*, 1993b) or they may be arranged with regular spacing as in *Boergesenia*, *Valonia* and other green algae (McNaughton and Goff, 1990; Shihira-Ishikawa, 1987). McNaughton and Goff (1990) pointed out an interesting correlation between the distribution of nuclei and the occurrence of cytoplasmic streaming: random distribution of nuclei leads to asynchronized mitosis and conspicuous cytoplasmic streaming, whereas regular spacing of nuclei coincides with a lack of cytoplasmic streaming but synchronized mitosis. The synchronous division cycle in *Boergesenia* was investigated by microspectrophotometry of fluorescently labeled DNA in combination with the monitoring of bromodeoxyuridine incorporation during S phase (Motomura, 1996). This study showed that almost all nuclei throughout the cell remain in the G_1 phase after completion of mitosis and that nuclei in several patches in the cell simultaneously enter the S phase for 12 h followed by 2 h of G_2 phase to complete one cell cycle.

Goff and Coleman (1987) measured the density of almost hexagonally arranged nuclei in the cytoplasm of the red alga *Griffithsia*, which exhibits a life history, where isomorphic alternation of haploid and diploid generations occurs. The nuclear density in haploid cells was twice as much as that of diploid cells, resulting in the maintenance of about the same amount of nuclear DNA per unit volume of cytoplasm despite different ploidy levels. On the other hand, the regulation of DNA content per volume of cytoplasm is less tightly regulated in the giant cells of green algae (Kapraun and Nguyen, 1994). Experiments using cytoskeletal inhibitors

have generally shown that the microtubule cytoskeleton may play an important role in maintaining the regular spacing of nuclei in the multinuclear cells of giant-celled algae (*Griffithsia* [Goff and Coleman, 1987], *Boergesenia*, *Valonia*, *Valoniopsis*, and *Ventricaria* [McNaughton and Goff, 1990]). Microtubules appear to function as spacers keeping nuclei from approaching each other. Even in some giant algae with spectacular cytoplasmic streaming such as *Acetabularia*, there are phases in the life cycle, when this principle function of microtubules prevails (Menzel *et al.*, 1996).

2.3. Motility of organelles

Movement of intracellular structures and cytoplasmic streaming are readily observed under a light microscope in many giant-celled algae. Because of the large size of the cell, such motility often results in long-distance transportation of organelles and cytoplasm surpassing that seen in cells of normal size by orders of magnitude. In fact, a number of studies on protoplasmic motility in giant-celled algae have been carried out, especially with the focus of correlating the motility with cytoskeletal elements such as microtubules and actin filaments and their associated motor proteins (La Claire, 1992; Menzel, 1996; Menzel *et al.*, 1992; Shimmen and Yokota, 1994). The most intensely studied experimental models were the internodal cells of characean algae, where endoplasm (an inner layer of peripheral protoplasm) including nuclei and other organelles streams at an extremely high velocity (more than 50 $\mu m\ s^{-1}$) along actin bundles (Kamiya, 1981; Williamson, 1992). The actin bundles are arranged in parallel on the inner surface of the cortical layer of cytoplasm along the regularly arranged chloroplasts. The required components and the regulatory mechanisms of the motility have been investigated extensively in many studies using the entire cells and membrane-permeabilized (perfused) cell models, and reconstituting the organelle motility in the perfused cells with exogenous proteins (Kamiya, 1986; Shimmen and Yokota, 1994; Wasteneys *et al.*, 1996; Williamson, 1992). More recently, *Chara* myosin has been characterized at the molecular level and dubbed ''the fastest myosin'' on earth because of its unique properties (Morimatsu *et al.*, 2000). For a comparison of *Chara* myosin with higher plant myosins see Shimmen and Yokota (2004).

In other giant algae exhibiting spectacular forms of organelle motility, the distinction between endoplasm and cortical cytoplasm is not as clear as in the characean algae. Two different types of motility occur along the long axis of the vegetative cell of *Acetabularia* (Koop and Kiermayer, 1980a; Menzel, 1994): slow movement (1–2 $\mu m\ s^{-1}$) of chloroplasts and rapid movement (3–11 $\mu m\ s^{-1}$) of the ''headed streaming band (HSB),'' a colorless cytoplasmic mass consisting of an amoeba-like head and a long, thin tail. Involvement of other structures such as chloroplasts, polyphosphate bodies, and secondary nuclei in HSB motility is often observed and this results in an

apparent increase in velocity of these organelles (Koop and Kiermayer, 1980a). Inhibitors of microtubules have no effect; however, inhibitors of the actin cytoskeleton have a strong effect. For example, cytochalasin B reversibly inhibits both types of organelle motility, indicating that these protoplasmic movements depend on the actin cytoskeleton (Koop and Kiermayer, 1980b), which is bundled and arranged in parallel arrays along the long axis of the cell (i.e., Menzel, 1986; Sawitzky *et al.*, 1996). In addition, rapid acceleration of chloroplast movements has been reported during the photoavoidance response from high-intensity light irradiated in a limited region of the cell in this alga (Mine *et al.*, 2002). In this case, there is no change in the arrangement of actin bundles before and after the photoavoidance response, but the rapid chloroplast motility is also inhibited by the treatment with cytochalasin D. This suggests that this movement is based on the motility along the cytoplasmic actin bundles (Mine *et al.*, 2002). Interestingly, another inhibitor of the actin cytoskeleton, the cyclodepsipeptide jasplakinolide, which promotes actin polymerization, also inhibited chloroplast motility and headed streaming band movement, indicating that the actin cytoskeleton undergoes dynamic reorganization to maintain long-distance motility tracks (Sawitzky *et al.*, 1999).

Motility of chloroplast and other organelles has been also studied with reference to arrays and function of cytoskeletal elements in *Bryopsis* (Menzel and Schliwa, 1986a,b) and *Caulerpa* (Menzel, 1987; Menzel and Elsner-Menzel, 1989c). In the gametophytic cells of *Bryopsis*, both actin filaments and microtubules are densely arranged in the longitudinal direction, and the former surrounds each chloroplast as though the filaments form a ''basket'' (Menzel and Schliwa, 1986a). Chloroplasts move along the longitudinal axis in *Bryopsis* and this motility is inhibited by either colchicine or cytochalasin D, indicating the importance of the organization of both microtubules and actin filaments in the chloroplast movements (Menzel and Schliwa, 1986b). On the other hand, movements of mitochondria and other small organelles appeared to rely entirely on the microtubule cytoskeleton and are most likely powered by a microtubule-dependent motor proteins (Menzel and Elsner-Menzel, 1989a). An interdependence between microtubules and actin filaments has also been shown for blue light-induced chloroplast movement in the protonema of the moss, *Phycomitrella patens* (Sato *et al.*, 2000), whereas chloroplast photoorientation movement in higher plants is dependent solely on actin (Takagi, 2003).

Motility of chloroplasts and amyloplasts in the giant coenocytic cell of *Caulerpa* is differentially affected by cytoskeletal inhibitors depending on the type of movement (Menzel and Elsner-Menzel, 1989c). Most chloroplasts are located in the cortical cytoplasm alongside both microtubules and fine actin filaments (Menzel, 1987), and they are not motile except in the case of photoavoidance response from local irradiation of UV light. This motility of chloroplasts is not blocked by microtubule inhibitors but inhibited by

cytochalasin D, whereas microtubule inhibitors block the streaming of amyloplasts, which were colocalized with thick bundles of microtubules in the subcortical layer of protoplasm (Menzel, 1987; Sabnis and Jacobs, 1967). In *Chlorodesmis*, which is a member of the sister family Udoteaceae within the Caulerpales, it has been shown that the amyloplasts are dorsoventrally organized, and that the flattened dorsal side, which is closely apposed to a microtubular bundle, is structurally reinforced by an intraplastidal ribbon of fibrils (Menzel, 1985). It is conceivable that microtubule-dependent motor proteins are attached to the outer surface of the ventral side mediating long-distance transport along the microtubule bundle. Manabe and Kuroda (1984) had already seen periodical crosslinks between the amyloplast membrane and microtubules in ultrathin sections of *Caulerpa* and suggested that these crosslinks represent dynein, because the periodicity had a maximum between 23 and 28 nm, reminiscent of the spacing of flagellar dynein.

3. Cytoskeletal Organization During Morphogenesis

In general, cell morphogenesis requires intracellular polarity and/or motility based on cytoskeletal elements such as actin filaments and microtubules (Fowler and Quatrano, 1997; Klymkowsky and Karnovsky, 1994; Madden and Snyder, 1998; Mathur and Hülskamp, 2002; Menzel, 1996; Verde, 1998; Volkmann and Baluska, 1999; Wasteneys, 2000). The cytoskeletal elements may at least function in directional transportation of materials used for the morphogenetic reshaping of the inner architecture of the cell. Visualization of the spatial arrangement of the cytoskeletons in the cells is of fundamental importance for understanding the functional relationship between the cytoskeletal structures and cell morphogenesis. Microtubules can readily be visualized by transmission electron microscopy (TEM) in ultrathin sections and have been studied as early as 1967 in the giant-celled algae (Sabnis and Jacobs, 1967). Fine structure work was originally less successful with respect to the visualization of actin filaments except in those algae with extremely prominent and tightly packed actin bundles such as the characean algae (Pickett-Heaps, 1967). However, fluorescently labeled phallotoxins as specific probes for F-actin and heterologous antibodies capable of recognizing plant cytoskeletal proteins became invaluable tools for the study of cytoskeletal structures at the light microscopic level (Goodbody and Lloyd, 1994). Because of the relatively lower resolution and bundling artifacts (Okuda *et al.*, 1990b) the latter technique cannot distinguish individual microtubules, even less so single

actin filaments, but it enables researchers to observe overall arrangement of actin filament bundles and microtubule structures in cells more easily.

Giant-celled algae, however, pose a special problem. Because the cells are large in size they are most often impossible to handle and mount in one piece. Such difficulty has been overcome by the development of microdissection techniques, by which cells are cut open during fixation and suitable slices are processed individually such as in the case of *Caulerpa* (Menzel, 1987), where cell wall trabeculae form an internal scaffold. Or, as in most other cases, cells are cut open during fixation, which facilitates quick entry of the fixative, and subsequently the thin layer of cytoplasm is removed either in parts or as a whole from the cell wall and processed further. This technique was first used on *Bryopsis* (Caulerpales; Menzel and Schliwa, 1986a) and later adapted to *Acetabularia* (Dasycladales; Menzel, 1986) and to *Boergesenia* (Siphonocladales; La Claire, 1987) and a number of other siphonoclad algae including *Valonia* and even *Hydrodictyon* (Chlorococcales) (McNaughton and Goff, 1990). Characean cells have most often been fluorochrome-labeled by perfusion/fixation or by microinjection (summarized in Wasteneys *et al.*, 1996). All these various methods were aimed at overcoming the cell wall barrier and enabled experimenters not only to introduce antibodies into the cytoplasm without perforating cell walls by enzymatic digestion, which is usually necessary for multicellular tissues with cell walls, but also to prepare sufficiently large flattened cytoplasmic sheets to observe the entire area of interest where the morphogenetic event has occurred. Only in one case, that of the giant-celled red alga *Griffithsia*, has a modified version of an enzyme digestion regime been adapted to introduce fluorochrome-labeled phallotoxins into the cells (McDonald *et al.*, 1993).

Using fluorescence microscopy in combination with high-resolution observation of microtubules and other subcellular structures in a specific region of the cell by TEM, a variety of studies have been published on the arrangement of cytoskeletal elements in morphogenetic phases of giant-celled algae. Pharmacological experiments using inhibitors of actin filaments and microtubules often complement those studies in order to clarify the function of the cytoskeletal element(s) in cell morphogenesis. In this chapter, we review selected examples of such studies with description of the process of morphogenetic events during vegetative and reproductive phases in giant-celled algae as well as wound-healing reactions that are of particular importance in the absence of cross walls in the giant-celled algae.

3.1. Vegetative morphogenesis

Phases of vegetative growth, cell shape differentiation, and division of the giant cells are treated as cell morphogenesis, whereas generative phases leading to the formation of reproductive structures and further to the liberation of gametes are treated as reproductive differentiation. As in

other organisms, both phenomena involve often dramatic intracellular reorganization closely related to intracellular motility and cytoskeletal dynamics in the giant-celled algae.

3.1.1. Cell growth

Cell growth is roughly categorized into localized growth, where cell expansion occurs polarly in a specific region of the cell, and diffuse growth without such spatial localization of a growing region. In the former, two different growth modes, tip and intercalary growth, have been observed in the giant-celled algae (Fig. 2.1). Although many questions remain open, there is a relationship between intracellular arrangements of cytoskeletons and spatially localized growing regions. This is particularly evident in the case of tip growth, but also in intercalary (band) growth the importance of the cytoskeleton is indicated at least by the effect of cytoskeletal inhibitors.

3.1.1.1. Tip growth Tip growth is the type of cell growth found in only a few cell types of higher plant cells such as pollen tubes and root hairs (Carol and Dolan, 2002; Dumais *et al.*, 2004), in which the expansion of the cell is limited to the dome-shaped end of the cylindrical cell, whereas intercalary growth in higher plant cells has rarely been addressed. In the lower plants and algae, including giant-celled algae, both intercalary growth and tip growth occur much more frequently. Because the extension rate of the

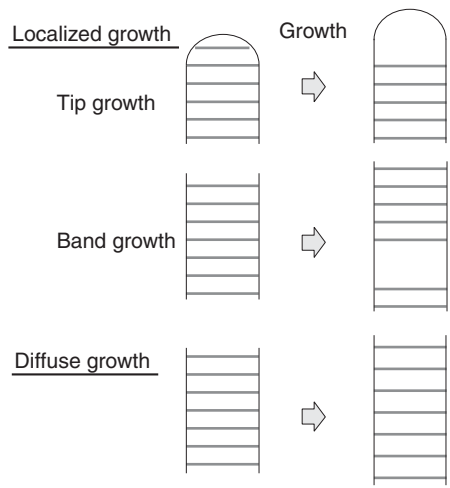

Figure 2.1 Patterns of cell growth in giant cellular algae. Thick, gray lines mark the cell surface to make the local surface expansion clearly discernible. In tip growth, only the cell surface in the domed apical region expands whereas growth occurs only at a narrow, transverse zone of the cell in band growth. In diffuse growth, cell surface expands uniformly along the entire length of the cell.

local cell surface is easily measured by continuous microscopic observations of growing cells labeled on their surface with resin particles, there are many examples of early studies analyzing the regional growth distribution around the tip-growing region in giant-celled algae, for example, in apical cells of *Nitella* nodal side branches (Green and King, 1966), *Nitella* rhizoids (Chen, 1973), *Chara* rhizoids (Hejnowicz *et al.*, 1977), and *Vaucheria* cells (Kataoka, 1982). In characean rhizoids and protonemata it has been shown that the growing tip is completely free of microtubules, and growth and gravitropic bending are entirely dependent on vesicle transport and spatial control of exocytosis and endocytosis based on the actin cytoskeleton (Braun and Limbach, 2006).

This is different in *Bryopsis* and other closely related members in this family. In the coenocytic thallus of the *Bryopsis* gametophyte organelle transport depends on both longitudinally arranged microtubules and bundles of actin filaments (Menzel and Schliwa, 1986a; Hishinuma *et al.*, 1997). Microtubule inhibitors such as colchicine and griseofulvin inhibit organelle movements, but they also inhibit cell growth, eventually causing an abnormal morphology of the thallus with numerous cell protrusions (Mizukami and Wada, 1983). Similar inhibitory effects are obtained by subjecting cells to high pressure or low temperature that is expected to destroy microtubules. These results suggest that the microtubule cytoskeleton is involved in the mechanisms of tip growth and apical morphogenesis probably through the intracellular transport system (Mizukami and Wada, 1983). Because both cytoskeletons interact closely with one another, which was shown *in vivo* (Menzel and Schliwa, 1986b) and in cytoplasmic exudates (Menzel and Elsner-Menzel, 1989a), it should be conceivable that they also maintain this cooperative interaction in the apical growth region supporting tip growth. However, further experiments employing actin inhibitors such as latrunculin B and cytochalasin D in conjunction with proper labeling techniques of cytoskeletal elements and endomembrane compartments should clarify this point. Evidence of the involvement of both the actin cytoskeleton and microtubules in the initiation and maintenance of polarized growth has been provided for the large-celled *Fucus* zygote, as it begins to form a rhizoidal outgrowth (Corellou *et al.*, 2006; Hadley *et al.*, 2006).

Actin and microtubule involvement in tip growth and organelle motility is also seen in the giant-celled xanthophyte *Vaucheria*. Thick bundles of microtubules, which are shorter than the longitudinally arranged microtubules in *Bryopsis*, are present in the peripheral cytoplasm, and they are apparently associated with nuclei at their anterior ends (Ott and Brown, 1972; Takahashi *et al.*, 2001). These bundles, termed microtubular probes (Ott, 1992), could be a functional element in nuclear movement, that is, they could act like a towing bar in front of a carriage. They run parallel to the long axis in the cylindrical, basal portion of the cell, whereas they are arranged randomly in the dense protoplasm of the tip-growing region in the

domelike apical end of the cell (Takahashi *et al.*, 2001). Kataoka (1982) reported that colchicine treatment causes changes in the polarity of cell surface expansion, from isotropic to transversely anisotropic, again indicating that the microtubule system plays an important role in the proper orientation of tip growth. In contrast, cells in the vegetative phase of *Acetabularia*, before meiosis, do not contain microtubules as shown by immunofluorescence microscopy (Menzel, 1986) and even tubulin proteins are not expressed as proven by Western blots (Menzel, 1994). Both techniques have shown the continuous presence of actin molecules in the cytoplasm and bundles of actin filaments that run in longitudinal directions in the cortical cytoplasm of the stalk instead of microtubules (Menzel, 1986, 1994). Improved visualization of actin filaments, using microwave-aided fixation, shows that the axial actin bundles extended to and converged at the tip of the growing apex (Sawitzky *et al.*, 1996). Data obtained on the effects of various cytoskeletal inhibitors on the tip growth in this alga (Sawitzky *et al.*, 2003) prove that the function of actin cytoskeletons is critical for cell growth in this alga. Microtubules do occur at a later stage in development, just before meiosis around the giant, primary nucleus and they remain associated after meiosis with the secondary nuclei as they move from the rhizoid to the cap rays. However, these microtubules do not have a function in the motility of the organelles; rather, they function as an anchoring device, when these nuclei assume their final position in the cap rays (Menzel *et al.*, 1996).

The polarity of microtubules is closely related to the polarity of tip growth in gametophytic cells of *Bryopsis* with the thallus tip at one end and the rhizoidal pole at the other end. The polarity of microtubules, which run longitudinally through the cortical cytoplasm, was determined in cross sections by TEM examination after performing the *in situ* synthesis of B-microtubules, so-called hooks (McIntosh and Euteneuer, 1984). It was shown by the uniform counterclock-wise direction of hooklike appendages on native microtubules that they are oriented with their plus ends toward the apical end of the cell (Hishinuma *et al.*, 1997). In addition, when two cell fragments of *Bryopsis* including a thallus tip were excised from different individuals and grafted, a rhizoid emerges from the junction. Similarly, two rhizoidal fragments form a thallus tip at the junction. Thus the polarity in the longitudinal microtubules is largely consistent with the local polarity of the cell, directing the plus end of microtubules toward the thallus tip. On the basis of these results and those from related experiments, it is suggested that the endogenous polarity of the cell is maintained fundamentally by the structural polarity of the microtubules (Hishinuma *et al.*, 1997).

The microtubule arrangement in the siphonoclad green algae is principally different from that in the caulerpalean green algae. Here the microtubules are arranged in regularly spaced, parallel arrays right underneath the plasma membrane and apparently do not support organelle movement, in fact, neither

chloroplasts nor nuclei are motile in this group of algae. There have been several reports showing dynamic changes in the arrangement of microtubules during the initiation and development of new growing tips, which are induced experimentally or formed spontaneously. For example, in the green alga *Chamaedoris*, the cylindrical cell exhibits polar elongation, which is probably not equivalent to tip growth as seen in the caulerpalean giant-celled algae and probably also unlike that in characean algae and tip-growing cell types of higher plants. Microtubules in the peripheral cytoplasm are arranged longitudinally in the cylindrical portion of the cell, whereas a meridional arrangement of microtubules is observed in the domelike apex of the cell (Okuda *et al.*, 1993a). Incubation of cells in culture medium containing the microtubule inhibitor amiprophos methyl (APM) reversibly suppresses cell elongation. Prolonged treatment with APM completely depolymerizes cortical microtubules as confirmed by immunofluorescence microscopy. Recovery of such cells in culture medium without APM causes the formation of additional growing tips along the lateral side of the cell. Before protrusion of these new tips, a dense aggregation of chloroplasts is formed in the region, where the new tip will be formed, and a radiating array of cortical microtubules is developed in this region. This radiating array of microtubules eventually becomes the meridional microtubule system, when the cell surface protrudes (Okuda *et al.*, 1993a).

Meridional arrangement of microtubules is also found in the initiation of a growing tip during cell regeneration from protoplast in the siphonoclad green alga *Boodlea* (Okuda and Mine, 1997). The longitudinal microtubule array in the intact *Boodlea* cell is transformed into a random arrangement of wavy microtubules on the formation of a protoplast by extrusion. During cell wall development, cortical microtubules begin to show a meridional arrangement in the spherical cells, before bipolar protrusion of both thallus tip and rhizoid tip occur (Okuda and Mine, 1997). As indicated in these studies, the meridional arrangement of microtubules is closely related to the initiation and maintenance of tip growth.

Spontaneous branching of *Vaucheria* produces a sparsely branched filamentous cell, but additional branch formation can be induced in regions where blue light is locally applied (Kataoka, 1975; Takahashi *et al.*, 2001). Takahashi *et al.* (2001) investigated changes in the arrangement of microtubules during the artificially induced branch formation process, and showed that the accumulations of chloroplasts and nuclei result in the thickening of protoplasm in the irradiated region. According to this study, bundles of microtubules originally arranged in longitudinal direction become accumulated together with nuclei in these irradiated regions, but then are shortened and scattered around in the thickened cytoplasm. The microtubule bundles are finally reorganized in longitudinal orientation from the subapical to the basal portion of the newly formed branch. Besides these obvious changes in the microtubule system, actin filaments also appear to

play a role in protoplasmic accumulation required for branch formation, because both APM and cytochalasin A inhibit branch initiation and in both cases microtubule bundles are destroyed and concomitantly nuclear accumulation is inhibited (Takahashi *et al.*, 2001). Therefore, this may be taken as another example, where microtubules interact with the actin filament system.

3.1.1.2. Band and diffuse growth Band growth is a less common mode of localized cell growth and is known in some uniseriate, cerameacean red algae including the giant-celled *Griffithsia*, but also other red algal genera of normal cell size (Waaland, 1990). As shown by a unique method using a fluorescent cell wall dye, longitudinal expansion of the cell surface occurs only along a narrow transverse band usually situated in the basal half of the cell. Although fluorescent labeling of cytoskeletons was quite successful in another cerameacean alga (Garbary and McDonald, 1996), detailed information of the arrangement of cytoskeletons is only partially obtained for the giant-celled alga *Griffithsia* (Garbary *et al.*, 1992; Russel *et al.*, 1996). According to these studies, actin filaments form a cortical meshwork throughout the intercalary cells, whereas microtubules arrange longitudinally. The longitudinal arrangement was denser in the basal region where band growth occurs, indicating a possible spatial relationship between microtubules and cell wall deposition during band growth (Garbary *et al.*, 1992).

Cylindrical internodes of *Chara* and *Nitella* have been used for studies of cell growth with special reference to the cell wall extensibility (see Section 4.1.3.2). These cells expand in both longitudinal and transverse directions (Richmond, 1983) and, in terms of the amount of extension, longitudinal growth is predominant in these cells and has been the main object of growth measurements (Green *et al.*, 1971; Metraux *et al.*, 1980; Proseus *et al.*, 2000). In these algae, transverse regions of the internode cells, around which the environmental pH is high, have been known as alkaline bands (Shimmen and Yamamoto, 2002). The analysis of regional expansion in the living cells and isolated cell walls of *Nitella* showed that longitudinal expansion was much weaker in the alkaline band region than in the remaining acidic regions of the cell (Metraux *et al.*, 1980). This peculiar growth mode is unlike genuine ''diffuse'' growth. Although the orientation of cellulose microfibrils is controlled by cortical microtubules (Baskin, 2001; Richmond, 1983; Richmond *et al.*, 1980), there is no spatial relationship between cytoskeletons and local growth activity, because the arrangement of both actin filaments and microtubules in the cell cortex is almost uniformly random throughout the length of the cell (Williamson, 1992). In addition, because an involvement of cytoplasmic streaming for the band formation has been suggested (Shimmen and Yamamoto, 2002), it is possible that the actin cytoskeleton indirectly plays a role in the localization of growing and nongrowing regions, or positioning of the alkaline band, respectively.

Although the distribution of regional cell expansion along the cell surface has not been investigated in the giant-celled algae consisting of single, globular to oblong cells, for example, *Boergesenia*, *Dictyosphaeria*, *Ernodesmis*, *Valonia*, and *Ventricaria*, these species seem to grow without restriction of cell expansion to specific region(s), because cells of these algae enlarge while maintaining the overall cell shape. Immunofluorescence microscopic observations of the microtubule cytoskeleton have been carried out extensively in these algae and indicate the presence of two microtubule systems throughout the cell: (1) cortical microtubules that run parallel to the long axis and converge at the apical end and (2) radial, perinuclear microtubules in the subcortical cytoplasm [*Dictyosphaeria* (Okuda *et al.*, 1997a); *Ernodesmis* (La Claire, 1987); McNaughton and Goff, 1990; *Ventricaria* (Shihira-Ishikawa, 1987)]. Whereas the cortical system could be involved in cell growth activities, the perinuclear microtubule systems lack any significant spatial relationship with cell growth activity. Attempts to visualize actin filaments by immunofluorescence microscopy in intact cells of *Ernodesmis* and *Boergesenia* have not been successful (La Claire, 1989); however, a fine, meshlike arrangement of actin filaments among chloroplasts has been seen in *Valonia* (Satoh *et al.*, 2000), again without a relationship to the cell growth.

3.1.2. Cell division

As in other organisms, it has been reported that in the giant-celled algae cell division involves diverse protoplasmic motility phenomena based on the dynamics of cytoskeletal elements and on motor protein activities. Besides the cytoskeletal organizations similar to those in normal cells such as spindles and contractile apparatuses for cytokinesis, in this chapter we highlight the role of the cytoskeleton with respect to spatial localization of division areas, protoplasmic aggregations, septum formation, and cell polarity in giant-celled algae. Cell division modes mentioned below include symmetrical and asymmetrical divisions apparently analogous to normal cell divisions, and two division modes, lenticular cell formation and segregative cell division, quite characteristic in giant-celled algae (Fig. 2.2).

3.1.2.1. Symmetrical or asymmetrical division Symmetrical cell divisions occur in the nonbranched, uniseriate alga *Chaetomorpha* (Kornmann, 1969; Okuda and Mizuta, 1987). After elongation by twice the length of the postmitotic daughter cell, the individual cell is divided equally by the formation of a transverse septum. Treatment with colchicine in moderate concentrations causes transverse expansion of the cell, and at the same time, cell division is suppressed (Okuda and Mizuta, 1987). However, colchicine has no effect on microtubules in this case. Because the arrangement pattern of cellulose microfibrils before and after colchicine treatment has not been modified, it may be concluded that cell expansion is due to

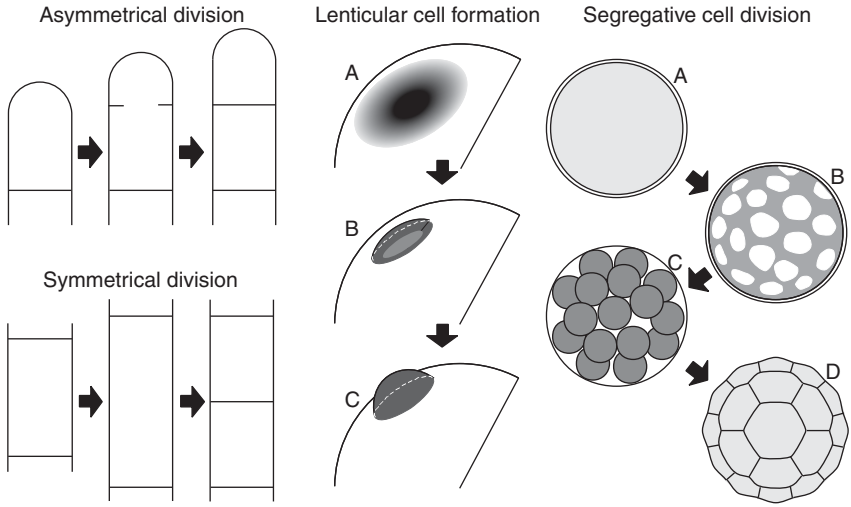

Figure 2.2 **Patterns of cell division in giant-celled algae. Asymmetrical and symmetrical divisions are apparently similar to those in normal organisms. In lenticular cell formation, aggregation of protoplasm (A), and centripetal septum formation beneath the protoplasm (B) are followed by growth of the separated cell. During segregative cell division in a globular cell such as *Dictyosphaeria*, thin peripheral protoplasm (A) aggregates into thick reticulate protoplasm (B), which is eventually torn off into many small, spherical masses (C). Each mass regenerates a cell wall and expands to tightly fill the entire surface of the parent cell (D).**

loosening of the fibril network of the cell wall that is enhanced by colchicine in this alga. The antimicrotubule action of colchicine in plants has been known for a long time, although more potent, plant-specific microtubule inhibitors are commonly in use (Bajer and Mole-Bajer, 1986).

Unequal cell division occurs in the apical cells of *Acrosiphonia*, another uniseriate, sparsely branched multicellular, giant-celled green alga. Before transverse septum formation in the upper portion of the apical cell, nuclei move toward and accumulate in the division zone and immunofluorescence studies have shown that microtubules are involved in this process (Aruga *et al.*, 1996). Microtubules, which are originally arranged in parallel arrays in the longitudinal direction, are rearranged and transformed into a thick band in the transverse direction at the site where septum formation will occur, and protoplasm is accumulated in the transverse zone around the microtubule band, where a bidirectional mitotic wave along the longitudinal cell axis is initiated. Finally, furrowing and centripetal septum formation take place. Unlike the actin-based contractile cleavage furrow that is typical of red and brown algae, and occurs also in some green algal lineages, the cleavage process in *Acrosiphonia* remains tightly associated with the equatorial microtubule band, which begins to shrink as the furrow proceeds

inward (Aruga *et al.*, 1996). The exact role of these microtubules has not been studied in detail; however, it appears as if the microtubule band guides the ingrowing cleavage furrow by an as yet undetermined mechanism.

3.1.2.2. Lenticular cell formation Lenticular cell formation in the siphonoclad green algae is a cell division mode that is extremely asymmetric. In *Valonia*, the first sign of lenticular cell formation appears as an accumulation of chloroplasts, which eventually expands into a dark green circular patch of thick protoplasm (protoplasmic disk). After the centripetal formation of a septum beneath the thickened protoplasm, the freshly separated cell domain bulges out and grows into a large clavate cell. In the early stage of chloroplast accumulation, cortical microtubules form an aster-like array radiating from the center of the protoplasmic disk toward its circumference. During subsequent stages of the process, this cortical microtubule organization changes progressively into a random arrangement (Okuda *et al.*, 1997b). Continuous APM treatment of the cell during the stage of protoplasmic disk growth results in the dispersion of the accumulated chloroplasts, implying that the microtubules may restrict chloroplasts in this area. Interestingly, because a bulge in the cell surface at the original position of the protoplasmic disk occurred during APM treatment (see Fig. 20.6H in Okuda *et al.*, 1997b), cell surface expansion seems to take place independently from the perturbation of intracellular organization and microtubules should not be required for bulge formation.

3.1.2.3. Segregative cell division Segregative cell division is another cell division mode characteristic in some siphonocladalean algae such as *Siphonocladus*, *Dictyosphaeria*, *Struvea*, and *Chamaedoris*. During the division, the protoplasm of the cell is gradually aggregated, forming thick reticulate protoplasm with many ''holes,'' from which protoplasm is excluded (Enomoto and Okuda, 1981; Enomoto *et al.*, 1982). Unlike the situation in *Valonia*, discussed above, portions of protoplasm are torn off at the narrower segments of this protoplasmic network, instantly forming many spherical protoplasts inside the parent cell wall. Further development of the separated protoplasts varies according to species (Enomoto and coworkers in Hori, 1994). Fluorescence microscopic observations of microtubule organization during segregative cell division in *Dictyosphaeria* have shown that the arrangement of two microtubule systems, cortical and perinuclear, is not directly related to the protoplasmic movement, but it does define the polarity of each derivative cell (Okuda *et al.*, 1997a). Thus the contraction of protoplasm during segregative cell division in this alga should be governed by motility systems such as those based on the actin cytoskeleton, rather than microtubules.

3.2. Reproductive morphogenesis

The reproductive development of the giant-celled algae includes a variety of morphogenetic patterns during sporogenesis, gametogenesis, and cyst formation.

3.2.1. Sporogenesis and gametogenesis

In spite of the large size of the vegetative cells, reproductive cells such as spores, zoospores, and gametes are similar in size to those of other algae. In the charophytes *Chara* and *Nitella*, the xanthophyte *Vaucheria* (Graham and Wilcox, 2000), the rhodophyte *Griffithsia* (Bold and Wynne, 1985), and some green algae (e.g., *Trichosolen* (Okuda, 1989; Okuda *et al.*, 1987)), small cells are cut off from the giant vegetative cell and differentiate into sporangia and gametangia, and the processes of sporogenesis and gametogenesis are similar to those in normal-sized algae. On the other hand, many of the giant-celled green algae, for example, *Bryopsis*, *Caulerpa*, *Chaetomorpha*, *Derbesia*, and *Dictyosphaeria*, produce reproductive cells in large sporangia or gametangia, which have been transformed directly from vegetative protoplasm in the confinement of the giant cells. Therefore, extraordinary reorganization of the protoplasmic architecture and concomitant reduction of cell size within the confinement of the giant parental cell occur during sporogenesis and gametogenesis, as well as during the numeral and spatial allocation of various organelles, which should be inherited to each reproductive cell.

In the *Bryopsis* gametophyte, entire side branches transform into gametangia (Burr and West, 1970). Okuda *et al.* (1993b) investigated the time course of the spatial distribution of nuclear density in the side branches of *Bryopsis* during their differentiation into gametangia and showed that the nuclear density increases remarkably in the greater part of the branch by induction of synchronous mitosis during a specific period after initiation of gametangial development. This phenomenon may represent an example of cellular differentiation processes providing the number of organelles corresponding to the number of reproductive cells to be produced in the cell. Regarding the spatial organelle allocation, to each reproductive unit, the availability of information is limited to some earlier studies. Ultrastructural studies on gametogenesis in *Bryopsis* (Burr and West, 1970), *Derbesia* (Wheeler and Page, 1974), and *Dictyosphaeria* (Hori and Enomoto, 1978) showed cleavage furrow formation as a means to separate a unit of forming gametes from the reminder of the protoplasm. In gametangia of *Trichosolen*, the growing cleavage furrow is led and possibly guided by microtubules (Okuda, 1989), whereas this has not been shown explicitly in the other species. Even more uncertain is the mechanism of cytoplasmic cleavage in the case of stephanokont zoospore formation in species such as *Bryopsis* and *Derbesia*.

3.2.2. Cyst formation

Mature cells of *Acetabularia* possess numerous cell projections around the cell apex, termed cap rays, which are formed after the stalk has reached its final length (exceeding 10 cm in some species of the genus). Reproductive development commences with the meiosis of the giant primary nucleus in the rhizoid and is followed by many rounds of mitosis. The secondary nuclei migrate through the stalk and enter the cap ray chambers. Each of the cap ray chambers becomes a special cell region for cyst formation. In early stages of cyst formation, the nuclei move along the cap ray, and, after some time, each nucleus becomes situated at a position equidistant from its neighbor. The cytoplasmic domain encircling each nucleus bulges out, and the spherical cytoplasm of a cyst is formed by concentric contraction of the edge around the cytoplasmic domain (Berger *et al.*, 1987; Menzel, 1994). As previously reviewed by Menzel *et al.* (1992) and Menzel (1994), it has been shown that two cytoskeletal elements, actin filaments and microtubules, exhibit distinctive functions in the dynamic motility of protoplasm during the cyst formation processes observed by immunofluorescence microscopy (Menzel, 1986; Menzel and Elsner-Menzel, 1990; Shihira-Ishikawa, 1989) and by inhibitory experiments (Menzel, 1988a; Menzel *et al.*, 1992, 1996).

Briefly, secondary nuclei first migrate along longitudinal actin bundles, and the number of nuclei in the cap rays increases as more and more arrive by migration from the stalk. Nuclear division in this phase is not prevalent, which is unlike the situation in *Bryopsis* (discussed above). In the migratory phase of the secondary nuclei, microtubules build a comet-like appendage at the trailing end of the nuclei. This assemblage of microtubules gradually increases and eventually is transformed into a radial array of perinuclear microtubules that functions as a bracing and anchoring device, defining the dimension of the future cyst domains and helping to maintain equal distance between neighboring domains (Menzel *et al.*, 1996). Later, when the cytoplasmic domain around the nucleus bulges out, thick bundles of actin filaments are formed along the edge of each domain. These may serve as contractile cytokinetic rings, which eventually split off the spherical cysts from one another (Menzel, 1988a; Menzel *et al.*, 1992). After separation, the microtubule systems serve as mitotic spindles as well as a circular microtubule band, lining under the future edge of the lid in the cell wall, which eventually opens to release the gametes from the mature cysts (Menzel and Elsner-Menzel, 1990).

3.3. Wound-healing reaction

The wound-healing reaction is particularly important for the survival of cells that have grown to the size of macroscopic individuals (La Claire, 1982b; Menzel, 1988b). In principle, the wound-healing reaction in these

algae includes rapid repair of cytoplasmic damage, preliminary closure of the opening in the cell wall inflicted by wounding, and the final regeneration of a new cell wall over the repaired surface of the cell (Fig. 2.3). Menzel (1988b) recognized six distinct steps in the response process including contraction of the cut edge of cytoplasm and closure of the cytoplasmic opening, in both of which cytoskeletal elements are potentially involved.

In *Ernodesmis*, the whole protoplasm quickly contracts after cutting off one end of the cell, forming a shrunken protoplast apart from the cut end (La Claire, 1982a,b), leaving microvillus-like structures on both surfaces, the contracting protoplasm, and the remaining cell wall, the latter of which are probably the remnants of cell wall–plasma membrane connections (Goddard and La Claire, 1993). The contraction can be induced even in detergent-permeabilized cells by elevating the Ca^{2+} level (La Claire, 1984). Cortical microtubules that are arranged parallel in the intact cell become wavy and displaced during contraction, which is interpreted as passive behavior and this is confirmed by the fact that APM and cold treatment,

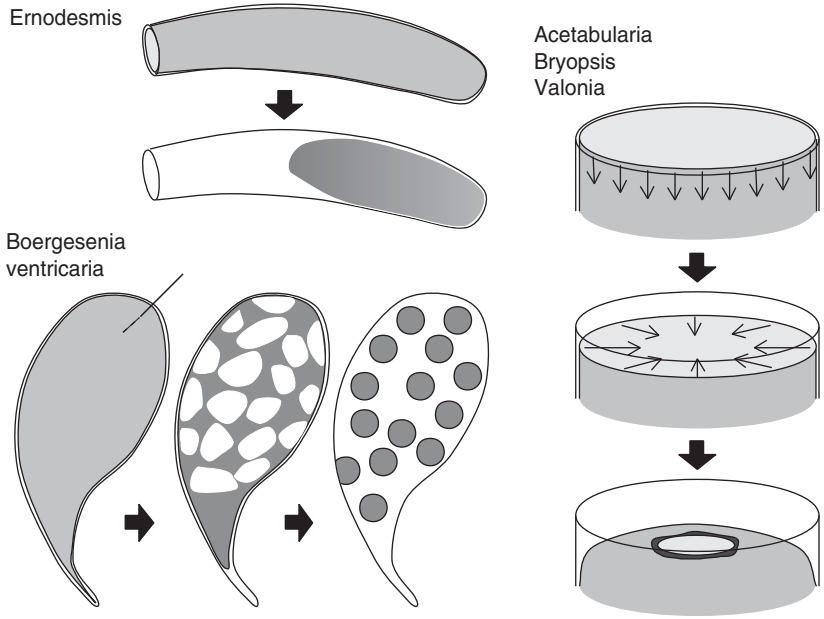

Figure 2.3 Patterns of wound-healing responses in giant-celled algae. In *Ernodesmis*, protoplasm quickly and vigorously contracts from the cut end of the cell. Mechanical stress induces aggregation of protoplasm, which is separated into aplanospores in some giant cellular green algae, for example, *Boergesenia* and *Ventricaria*. When a portion is cut off from the cell of *Acetabularia*, *Bryopsis*, and *Valonia*, the thin, peripheral protoplasm contracts to a lesser extent than in *Ernodesmis*, and the protoplasm is sealed by centripetal closure of its circular edge.

in which cortical microtubules are depolymerized, do not affect the wound-healing response (La Claire, 1987). In contrast, actin filaments are active components in the contraction process. In unwounded cells actin filaments cannot be detected by immunofluorescence microscopy but as contraction begins, they exhibit two patterns of distribution in the cytoplasm: a reticulate arrangement outlining chloroplasts and small vacuoles and longitudinal thick bundles near the cut end (La Claire, 1989). Interestingly, cytochalasins did not depolymerize actin filaments in this alga; however, erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) inhibited the formation of actin bundles and also the contraction completely. Although EHNA is not a bona fide actin inhibitor, these results have been interpreted as an indication for the involvement of the actin cytoskeleton in protoplasmic contraction and wound healing. And indeed, this has been confirmed by the demonstration that myosin is associated with the contracting actin arrays (La Claire, 1991).

Cell motility in some other siphonoclad giant-celled algae, for example, *Boergesenia* and *Valonia*, is different from that in *Ernodesmis*. In *Boergesenia*, cutting of a cell induces the formation of a reticulate protoplasm and subsequently numerous protoplasts form, which are sometimes designated as "aplanospores" (Enomoto and Hirose, 1972; La Claire, 1982b). These protoplasts later germinate to develop into a new generation of giant cells (Ishizawa *et al.*, 1979; and see below). In *Valonia* yet another mode of wound repair is encountered. Here the wounded protoplasm contracts locally at the wound site leading to a concentric closure of the protoplasmic layer right underneath the lesion in the cell wall (Satoh *et al.*, 2000), followed by final repair of the cell wall by the deposition of new wall layers along the surface of protoplasm. *Ventricaria* alters the wound-healing responses according to the size of the wound, which in turn affected the capability of the cell to regenerate its turgor: a small injury causes a local contraction of the wound opening in the protoplasm and turgor is restored, whereas a large injury induces the formation of aplanospores involving large portions of protoplasm so that the central vacuole becomes disfunctional. Each of the aplanospores develops a central vacuole and restores turgor individually (Nawata *et al.*, 1993).

The repair mechanism in the dasyclads appears to be similar to that in *Valonia* and has been studied in further detail. On wounding, the peripheral cytoplasm of *Acetabularia* retracts somewhat from the cut end, but then closes by concentric contraction of the circular edge of the cytoplasmic wound (Menzel, 1988b; Menzel and Elsner-Menzel, 1989b; Menzel *et al.*, 1992). Dense bundles and clusters of actin filaments are observed along the contractile edge of the wound, apparently formed by local dispersion and reorganization of the original axial actin bundles, which predominantly populate the unwounded cytoplasm (Menzel and Elsner-Menzel, 1989b). This reorganization takes place in response to calcium entry in a rather limited zone close to the wound edge, so that one can see the axial bundles

running right into the contracting edge. In all these examples of wound repair in the siphonoclad and dasyclad algae, microtubules are not involved in contractile movements (Menzel and Elsner-Menzel, 1989b; Satoh *et al.*, 2000).

Concomitant with the contraction processes going on in the wound protoplasm, a layer of a sealing material is formed. In the *Bryopsis* gametophyte, the wound-healing reaction is similar to that in *Acetabularia* and *Valonia*; however, unlike *Valonia* and different from *Acetabularia*, a bulky plug is formed at the wound (Burr and West, 1971; Menzel, 1988b). The proteinaceous precursor of the plug is produced in the cytoplasm secreted into the central vacuole and on wounding extruded through the cut end of the wound to be coagulated and hardened, thus sealing the cut end from the outside (Burr and West, 1971, 1972; Menzel, 1988b). A wound plug is also formed in the more delicate siphonous thallus of *Derbesia* and the corresponding giant-celled gametophytic phase, named *Halicystis*. Wound plugs are also present in the giant-celled dasyclads, although often less bulky than in *Bryopsis*. All of them contain a high amount of peroxidase, which appears to be associated with the plug precursor material stored in the central vacuole and becomes activated at the instance of wounding. The enzyme can easily be demonstrated by histochemical staining with peroxide and benzidine derivatives, but is also detectable in cell extracts by colorimetric assays (Menzel, 1980). Because dasyclads contain bicyclic, aromatic secondary metabolites, which possess antimicrobial activity and can be polymerized by the action of peroxidase (Menzel *et al.*, 1983), it has been suggested that in the dasyclads the enzyme has a protective function by hardening the wound plug material once it is extruded from the cut open cell, which occurs in a matter of minutes, before the wound is covered by a new cell wall. The same principal role of peroxidase can be assumed for the other cases mentioned above, except that the chemical nature of the cross-linked material may vary.

Vacuolar contents also appear to be important in the wound response of siphonoclads. The vacuolar contents in *Ventricaria*, which have been histochemically identified as sulfated polysaccharides, cover the newly formed protoplast during the early development of aplanospores, which have formed in response to wounding (Shepherd *et al.*, 2004). Although the biochemical nature of the extruded vacuolar material may be quite different, in basically every instance of wound response in the giant-celled green algae there is a phenomenon analogous to plug formation.

It is also noteworthy that the protoplasm of *Bryopsis* itself has extraordinary vitality. The protoplasm exuded by extreme wounding or when squeezed out from a cell, spontaneously aggregates to form a protoplasmic mass, which first repairs all damage to the plasma membrane and tonoplast so that turgor pressure can be restored and then forms a thin cell wall eventually becoming a viable cell, which subsequently reestablishes polarity

and regenerates into a normal *Bryopsis* thallus (Tatewaki and Nagata, 1970). The distinct requirements of the physiological condition for protoplasm aggregation and regeneration of the plasma membrane have been investigated in detail (Pak *et al.*, 1991) as well as the process of cell membrane formation (Kim *et al.*, 2001). Similar protoplasmic regeneration has also been observed in other giant-celled green algae [e.g., *Boodlea* (Okuda and Mine, 1997) and *Chaetomorpha* (Klotchkova *et al.*, 2003)]. Moreover, it has been shown that a new cell was regenerated when chloroplasts and protoplasmic components were reunified, after they had been separated by fractionated centrifugation (Kobayashi and Kanaizuka, 1977, 1985).

Giant internodal cells of the characean algae, which are members of a phylogenetic lineage of green algae distant from the marine siphonous green algae, also form wound plugs from preformed precursor material stored in the vacuole (Foissner, 1987), and they are capable of repairing smaller puncture wounds by localized exocytosis of callosic wall material. The latter activity is based on a meshwork of cortical actin filaments but is independent of microtubules (Foissner and Wasteneys, 1997).

The wound-healing response in the red alga *Griffithsia* is different in that it involves cell differentiation of adjacent cells, whereas the wounded cell is not repaired (Waaland, 1990; Waaland and Cleland, 1974). When an intercalary cell of the uniseriate filament of *Griffithsia* is injured or killed, two specialized cells (repair cells) are produced from the neighboring cells at the distal and proximal sides of the dead cell. These repair cells extend within the hollow cell wall of the dead cell toward each other and fuse to form a single cell bridging between the neighboring cells. This cell eventually replaces the dead cell, completing the wound-healing response. The involvement of cytoskeletal elements in this cell repair process has not been clarified yet, but cytoskeleton(s) may play a certain role at least in the directional growth of the repair cells.

4. Physiological and Molecular Biological Aspects of Morphogenesis

4.1. Physiological factors controlling morphogenesis

The morphogenetic events in giant-celled algae mentioned in the previous chapter are affected by many internal and external physiological factors, some of which can be studied by changing environmental conditions during laboratory cultures. A number of such studies on the physiology of these morphogenetic events have been conducted utilizing unique traits of giant-celled algae, that is, typical morphogenetic events, which can be readily assessed. Here we mention selected examples of such studies on external,

physiological and ionic factors, as well as those related to cell walls and internal factors, such as hormones and signal transduction.

4.1.1. External factors

Under conditions of laboratory culture, it is not difficult to control external physical factors, such as light, temperature, and gravity, with certain precision, and many giant-celled algae respond to these factors by altering their morphology by growth and differentiation.

4.1.1.1. Vegetative growth Growth, including tip growth and diffuse expansion of the cell surface, can be controlled by light and temperature quantitatively, particularly by the intensity and quality of light. In earlier experiments on the germination of aplanospores of *Boergesenia*, Ishizawa *et al.* (1979) showed that the germination is temperature dependent, with an optimum of 30°C, and germination is promoted by irradiation of red light depending on its duration and intensity. Ohba and coworkers (Ohba and Enomoto, 1987; Ohba *et al.*, 1992) have cultured two varieties of *Caulerpa racemosa* under various light and temperature conditions. These varieties, which had been classified by the shape of the erect portions (shoot) of coenocytes, altered the shoot morphologies including those typical for both varieties and their intermediates depending on culture conditions. On the basis of this morphological plasticity, these varieties have been concluded to be ecophenes of the species (Ohba *et al.*, 1992).

In the internodal cell of *Chara* there are relationships between longitudinal cell growth, temperature, and artificially controlled turgor pressure (Proseus *et al.*, 2000). Lowering the temperature causes the turgor pressure of cells to decrease, resulting in the inhibition of cell growth. Cell growth is not rescued by artificially elevating the turgor pressure to its original level, but by returning to the original temperature, because a decrease in turgor pressure is apparently caused by metabolically affecting the osmotic potential of the cell (Proseus *et al.*, 2000). In the vegetative diploid cell of *Acetabularia*, several successive sets of hair whorls are produced from the growing cell apex (Berger *et al.*, 1987; Menzel, 1994). Whorl formation does not occur under red light, but is induced by pulse irradiation with blue light in a fluence-dependent manner (Schmid *et al.*, 1987). Moreover, the spacing between these hairs in the whorl, that is, the number of hairs per whorl, is controlled by temperature (Dumais and Harrison, 2000; Harrison *et al.*, 1981) and also by the Ca^{2+} concentration in the culture medium (Dumais and Harrison, 2000; Harrison and Hillier, 1985).

Vegetative growth in giant-celled algae is also affected by the direction of light and gravity. *Chara* rhizoids have been used as materials for such experiments expressing positive gravitropism (Sievers *et al.*, 1996). Statoliths, small vacuoles containing $BaSO_4$ crystals, are situated in the subapical cytoplasm in the tip-growing region of the rhizoid, involving the actin

cytoskeleton and myosin motor proteins (Braun, 2002). Limbach *et al.* (2005) have suggested that the perception of gravity requires statoliths to move and to make contact with receptors in the plasma membrane; however, the macromolecular nature of the receptor is not yet known. The phototropic growth responses of both thallus and rhizoid apices in the *Bryopsis* gametophyte, where positive and negative phototropic bending was observed, respectively, were studied by Iseki *et al.* (1995a,b). Although both modes of phototropic bending show action spectra with a peak at 467 nm, the growth pattern for bending was different between the two: the positive phototropic bending in the thallus apex was caused by different growth rates of the shaded side as compared with the irradiated side, whereas the negative bending in the rhizoid was brought about by subapical "bulging," on the shaded side (Iseki *et al.*, 1995a,b).

In *Vaucheria*, two distinct light-induced growth reactions, phototropic bending at the growing apex and, as already mentioned, branch formation in the nongrowing region of the cell, have been investigated by Kataoka and coworkers. Positive phototropic bending of the thallus apex is induced by blue light and achieved by bulging at the illuminated side of the apex (Kataoka, 1975), and this reaction is accompanied by an influx of extracellular Ca^{2+} ions (Kataoka, 1990). Branch formation in the nongrowing region of *Vaucheria* occurs 5–10 h after the beginning of localized illumination with blue light (Takahashi *et al.*, 2001). The direction in which the branch forms on the surface of the cylindrical cell is independent of the direction of light. On "wedgelike" illumination, where the width of the field of illumination is unequal, the branch is always formed on the side that received more light. Therefore, branch formation appeared to be not a tropic response but a dose-dependent response (Takahashi *et al.*, 2001). In addition, it has been reported that *Vaucheria* exhibits a gravitropic response, even though it has no obvious statolith-like structures (Gavrilova *et al.*, 1997).

The direction of (blue) light illumination also determines the direction of bipolar germination in regenerating protoplasts of giant-celled green algae [*Boergesenia* (Ishizawa *et al.*, 1979) and *Boodlea* (Okuda and Mine, 1997)]. In *Boodlea*, APM treatment appears to inhibit the light-induced determination of the growth axis (Okuda and Mine, 1997), indicating that the construction of the microtubule system is an important factor for the establishment of the growth axis.

4.1.1.2. Developmental and reproductive differentiation As previously reviewed (Berger *et al.*, 1987; Mandoli, 1998; Menzel, 1994), the giant diploid unicell of *Acetabularia* develops into a thallus with complex morphology, including vegetative structures such as rhizoid, upright stalk, hair whorls, growing apex, and cap rays. The sequence of vegetative morphogenesis in *Acetabularia* has been known since the seminal work of Hämmerling (1931) and, early on, he had discovered that cells regenerate

and form a new growing apex after amputation. Even if the entire anterior stalk portion including the cap is removed in fully developed cells, the remaining rhizoid repeats the cycle of vegetative morphogenesis without fatigue as long as the primary nucleus has not been destroyed (Hämmerling, 1932, 1934).

The cap holds the majority of photosynthetically active chloroplasts and serves an assimilatory function for several weeks before becoming a sporophore, and therefore it should initially be regarded a vegetative structure. Only after meiosis of the giant, primary nucleus in the rhizoid and after the hundreds of secondary haploid nuclei have been transported via the actin filament bundle system into the cap ray chambers, does the cap become a reproductive structure (summarized in Menzel, 1994). Therefore, the onset of the reproductive phase is not at the moment of cap formation as proposed by Mandoli (1998), but at the moment when the first identifiable precursors of the future reproductive agents are formed, that is, the secondary nuclei. In the course of cyst morphogenesis, each of these nuclei, as far as they have arrived in the cap ray chambers, gives rise to a cyst by a complex process of protoplasmic restructuring within the cap rays as described in Section 3.2.2. The cyst at the uninucleate state is the equivalent of an aplanospore and hence it is the virtual reproductive agent. Cysts usually complete their gametophytic development within the confinements of the cap rays, which consist of just two steps before gametes are released: (1) the formation of the lid (Neuhaus-Url and Schweiger, 1984) within the cellulosic cell wall around the cyst, which involves the microtubule system (Menzel and Elsner-Menzel, 1990), and (2) the production of pregametic nuclei by many rounds of nuclear division followed by the partitioning of the protoplasm and the formation of gametes. To establish this alga as a tractable experimental model for genetic analysis of development, Mandoli and coworkers have developed inbred lines (Cooper and Mandoli, 1999; Nishimura and Mandoli, 1992), among them mutant lines affected in vegetative and reproductive development (summarized in Mandoli, 1998). It has been known for a long time that morphogenesis in *Acetabularia* is dependent on the light quality (summarized in Schmid, 1984). The requirement of blue light for whorl initiation has been investigated in detail by Schmid and coworkers (1987), who have proposed that the influence of light on vegetative morphogenesis stems from blue light-sensitive mechanisms that selectively control stage-specific translation and differential degradation of enzymes (Nickl *et al.*, 1988). The blue light requirement for cap ray formation has also been studied in inbred lines of *A. acetabulum*, which had been synchronized for cap formation by amputation of the apex (Kratz and Mandoli, 1999).

It has been generally known that the reproductive differentiation of macroscopic algae is controlled by laboratory culture conditions such as light and temperature (Lüning, 1981) and some giant-celled algae have been

known to respond to such environmental condition(s) by controlling their reproductive development. For example, Okuda and Tatewaki (1982) have shown that the timing of the formation of gametangia in *Trichosolen* is controlled by a circadian rhythm, which is synchronized with an exogenous photoregeme.

4.1.2. Electrophysiological factors and ion influx/efflux

Because of the size of cells and the relative ease with which electrodes can be inserted, the giant-celled algae have provided excellent materials for electrophysiological studies, especially the characean algae, but also some siphonoclads and dasyclads (Hope and Walker, 1975). Here we mention some of these studies that are related to morphogenesis through the regulation of ionic conditions and turgor pressure in cells. Turgor pressure has been assumed to generate the tensile stress of the cell wall, which serves as the driving force of the cell wall expansion during cell growth in plants (Cosgrove, 1997); therefore, regulation of turgor pressure is an important factor of cell growth and morphogenesis. In giant-celled algae, the turgor pressure should be regulated by influx/efflux of ions into the vacuole, which occupies most of the volume of the cell (Bisson, 1995). As reviewed by Bisson *et al.* (2006), studies on many giant-celled green algae have shown that electrophysiological ionic responses occurred on changes in the external osmotic potential, in order to regulate the turgor pressure of the cell. However, it has also been suggested in *Valonia* that characteristic structures at the cell periphery and variations in the polysaccharide vacuolar contents may contribute to an attenuation of the external osmotic challenge (Heidecker *et al.*, 2003). The mechanisms for the ionic regulation of turgor are different according to the organism and the kind of osmotic stress (hyper- and hypotonic). In particular, remarkable differences have been reported between two well-studied taxa, characean algae and *Valonia/Ventricaria*; that is, the electrical potential difference between external medium and vacuole is slightly positive in *Valonia/Ventricaria* but negative in characean algae, and potential changes in response to osmotic stress are also quite contrasting. For example, electrical potential differences become more negative in characean algae but more positive in *Valonia/Ventricaria* in response to hypertonic stress (Bisson *et al.*, 2006).

As mentioned previously in Section 3.1.1.2, longitudinal cell expansion of the internodal cell of *Chara/Nitella* is suppressed in the region of the transverse alkaline bands (Metraux *et al.*, 1980). The alkaline band is generated by local efflux of OH^-, which might be delivered by cytoplasmic streaming to the site of band formation (Shimmen *et al.*, 2003). The alkaline environments generated around the OH^--extruding area of the cell may affect growth through changing the extensibility of the cell wall (see below). Thus the internode cells of these algae potentially serve as a good material for observation of cell elongation controlled by electrophysiological factors. Interestingly, although the mechanisms for the formation of alkaline bands

are not fully understood, it has been further shown that alkaline bands in *Chara* could be induced in the cell area, where chloroplasts were removed by artificial wounding (Shimmen and Yamamoto, 2002).

4.1.3. Synthesis and mechanical properties of the cell wall

Growth of cells covered with a cell wall involves expansion of the existing wall and deposition of new wall materials. Therefore, knowledge about constituents and synthesis of the cell wall as well as physical properties of the cell wall, such as extensibility and strength, in the growing cells should provide fundamental information for the understanding of the controlling mechanisms for cell growth.

4.1.3.1. Cell wall synthesis As in algae of normal cell size, it is known that the cell wall constituents in giant-celled algae exhibiting a heteromorphic life cycle may be quite different between haploid/diploid generations. For example, the wall of diploid giant cells of *Acetabularia* consists of mainly mannans and lacks cellulose, whereas the cell wall of the haploid cyst is composed of 80% cellulose and 20% mannan (Bachmann and Zetsche, 1979). The haploid erect thallus of *Bryopsis* has a cell wall made of xylan (Huizing *et al.*, 1979) that has been purified in a study by Fukushi and Maeda (1986), and the wall of the sporophyte is composed of mannan (Huizing *et al.*, 1979). Similar alternation of cell wall components was also confirmed in the related genus *Derbesia* (Huizing *et al.*, 1979).

Many giant-celled algae have cellulose as major constituents of their cell walls. Cellulose microfibrils are composed of molecular crystals of glucose, made by intermolecular hydrogen bonds among β-1,4-glucan chains, which provides the mechanical stiffness and tensile strength of the cellulosic cell wall. The cellulose-synthesizing enzyme is a multisubunit complex seen as a terminal complex (TC) in the electron microscope by the freeze–fracture method. TCs have also been discovered in giant-celled algae, for example, in *Boodlea* (Mizuta, 1985), *Nitella* (Hotchkiss and Brown, 1987), *Valonia* (Itoh, 1990; Mizuta and Okuda, 1987b), and *Vaucheria* (Mizuta and Brown, 1992), and consequently, cellulose is a major structural polysaccharide in their cell walls. The question is, however, whether the mechanism of cellulose fibril alignment is the same as in higher plants. It is widely accepted that cells growing anisotropically have cellulose microfibrils arranged perpendicular to the growth axis and that these microfibrils function as a restraining girdle allowing growth to occur in only one direction, that is, along the longitudinal axis (Baskin, 2005). As mentioned in Section 3.1.1.2, characean algae provide an example, among the giant-celled algae, of this growth pattern. For higher plants and the unicellular green alga *Closterium* (Giddings and Staehelin, 1991) it has been proposed that microtubules may guide cell wall fibril alignment (i.e., the fluid channel hypothesis). However, among the giant-celled algae there are examples that do not conform

with this hypothesis. For example, colchicine-treated cells of *Chaetomorpha* exhibit a shift in the expansion pattern from anisotropic to isotropic growth without any changes in microfibril alignment (Okuda and Mizuta, 1987). So, even though microtubules must be assumed to be involved in controlling the direction of growth, there is no obvious geometric relationship between them and the cellulose fibrils in the wall. As reviewed by Baskin (2001), there are also a number of exceptions to the fluid channel hypothesis, where no spatial relationship was observed between the arrangement of microtubules and cellulose microfibrils. Mizuta and Okuda (1987a) investigated microfibril arrangement in the growing tip of *Boodlea* and found that the cell wall is composed of layers with three kinds of fibril orientation alternating regularly from transverse to longitudinal and then oblique, whereas cortical microtubules are always arranged parallel to the long axis of the cell. Thus microfibril orientation is, at least in two of the three layers, not related to the constant longitudinal direction of cortical microtubules (Mizuta and Okuda, 1987a). Similar disagreement has also been reported in regenerating protoplasts in *Boodlea* (Okuda and Mine, 1997; Okuda and Mizuta, 1985).

Relationships between cortical microtubules and cellulose microfibrils have been studied extensively in the tip-growing giant cells of *Chamaedoris* (Mizuta *et al.*, 1989; Okuda *et al.*, 1990a, 1993a). As stated in Section 3.1.1.1, a radial array of cortical microtubules is formed before the formation of a new growing tip from the cell that recovered from depletion of microtubule by APM treatment (Okuda *et al.*, 1993a). This microtubule array eventually develops into the meridional microtubule system in the tip of a new branch, suggesting that the meridional microtubule arrangement is involved in the initiation and maintenance of localized growth in the cell apex. On the other hand, Okuda *et al.* (1990a) indicated that the cell wall of this alga is composed of three kinds of cell wall layers that differ in the orientation of microfibrils, just as in *Boodlea*. The individual layers are periodically initiated one after the other at the growing tip, and they are deposited basipetally. These observations indicate that the alternation of microfibril orientation in the cell wall is controlled at the apical growing region of the cell and once the direction of fibril orientation is established there, it is maintained as the front of TCs moves basipetally, disregarding the orientation of microtubules in these distal regions of the cell (Okuda *et al.*, 1990a). Microtubules are likely involved in the ordered shift in microfibril orientation, because their disruption by APM inhibited the regular alternation of microfibril orientation, resulting in a uniformly helicoidal pattern of deposited microfibrils (Mizuta *et al.*, 1989).

In the growing tip of the giant cell of *Vaucheria*, application of microtubule inhibitors, in this case colchicine, also alters the mode of cell expansion from isotropic to anisotropic (Kataoka, 1982); however, there are no obvious microtubules present in the cortical cytoplasm (Takahashi *et al.*, 2001) and the orientation of cell wall microfibrils is always random in cells

not treated with colchicine (Mine and Okuda, 2007). Cell growth pattern might not be controlled by the regulation of the orientation of cellulose microfibrils and cortical microtubules but may be regulated by the mechanical properties of the cell walls affected by cell wall matrix components in this alga, as reviewed in the following section.

Several examples of studies on the relationship between cell wall metabolism and developmental or physiological factors in giant-celled algae are available. Bachman and Zetsche (1979) reported that the activities of two enzymes, guanosine diphosphate mannose pyrophosphorylase and mannan synthase, necessary for the synthesis for mannan, the major constituent of the cell wall of *Acetabularia*, are high in the apical region of the tip-growing cell, and drop when the cell terminates cell growth. In *Nitella* internodal cells, the cell wall composition and sugar incorporation into the cell wall were compared among rapidly growing, slowly growing, and mature cells (Morrison *et al.*, 1993). As a result, the rate of cell wall synthesis became slow and the orientation of cellulose microfibrils changed from transverse to helicoidal as the cell matured, and cellulose synthesis became higher than synthesis of matrix polysaccharides such as pectin and xyloglucan in the secondary cell wall. Xyloglucan endotransglucosylase/hydrolase, which is assumed to act on xyloglucan chains, rendering cell walls extensible in higher plants (Nishitani, 1997), was reported in the growing region of *Chara* cells (van Sandt *et al.*, 2006), although the presence of this matrix polysaccharide in *Chara* is still questioned (Popper and Fry, 2003). From a series of well-designed experiments using living cells and an isolated cell walls in *Chara*, Proseus and Boyer (2005, 2006a) showed that turgor pressure moves polysaccharides from the periplasmic space into the cell wall and contributes to the gel formation in the cell wall by concentrating the polysaccharide.

Regenerating protoplasts have served as an interesting experimental system for studying the construction of cell wall architecture in higher plants (Shea *et al.*, 1989). As stated above, protoplasts of giant-celled algae are even better suited. They may readily regenerate and are obtained without enzymatic treatment by aplanospore formation, as in *Boergesenia*, or by spontaneous aggregation of protoplasm squeezed out of cells, as in *Bryopsis*, *Chaetomorpha*, and *Boodlea* (Kim *et al.*, 2001; Klotchkova *et al.*, 2003; Mizuta *et al.*, 1985; Okuda and Mizuta, 1985). These studies have unequivocally shown that a temporal cell covering with amorphous texture containing wall matrix components is formed initially around the protoplasm, before the main constituents of the cell wall (the same materials as those of their original cell wall) begin to be deposited.

4.1.3.2. Mechanical properties of the cell wall Extensibility of cell walls has usually been examined experimentally by the observation of stretching of tissue or cell wall segments under tensile stress or by the measurement of stress relaxation after the segments had been stretched to a fixed length

(Cosgrove, 1993). In the giant-celled green alga *Halicystis* the elastic modulus of the cell wall was correlated with ion transport and hydraulic conductivity of the plasma membrane (Graves and Gutknecht, 1976; Zimmermann and Hüsken, 1980). An earlier study on cell wall extension in *Ventricaria* reported that, in contrast to results in a higher plant, the extensibility promoted by acidic pH is not dependent on cell wall proteins but was mimicked by displacement of wall-bound Ca^{2+}, using a chelating agent (Tepfer and Cleland, 1979). The relationship between growth and tensile stress of the cell wall was analyzed in detail by measurement of surface extension and thickness of local cell wall in *Acetabularia* (von Dassow *et al.*, 2001). Besides these investigations, many studies on the mechanical properties of the cell wall in giant-celled algae have been carried out on diffuse- or intercalary-growing internodal cells of *Chara*/*Nitella* and, more recently, on tip-growing cells of *Vaucheria*.

A number of earlier studies using *Chara*/*Nitella* as experimental model have addressed the stress–strain relationship, creep, hysteresis, and anisotropy of cell wall extension by uniaxial (Probine and Preston, 1962) and multiaxial stress (Kamiya *et al.*, 1963) and stress relaxation (Haughton *et al.*, 1968). More recently it was shown that the cell wall of *Nitella* with transverse orientation of cellulose microfibrils exhibits a greater plastic extension in the longitudinal direction than in the transverse direction (Richmond *et al.*, 1980). However, when *Nitella* cells are treated with the antimicrotubule drug isopropyl *N*-phenylcarbamate (IPC), microfibrils in the inner wall layer became randomly oriented and cell walls also became extensible in the transverse direction (isotropical; Richmond *et al.*, 1980). This suggests that microtubules control the orientation of microfibrils in newly formed innermost layers, and that this layer is predominantly responsible for the mechanical property of the cell wall. Changes in microfibril orientation caused by IPC also affect the growth pattern in living cells (Richmond, 1983). In addition, infrared spectrometry has also been used for analysis of structure and orientation of specific constituents of the unstretched *Nitella* cell wall (Morikawa *et al.*, 1974) and the stretched *Chara* cell wall (Toole *et al.*, 2004).

Physiological, physical, and chemical factors changing the mechanical properties of cell walls have also been investigated with *Chara*/*Nitella* cells. Metraux *et al.* (1980) have compared the extension of cell walls isolated from young, growing cells of *Nitella* with that of cell walls from old, mature cells by applying multiaxial stress. As a result, plastic deformation in the young cell wall was markedly increased at the pressure equivalent to the actual turgor pressure, whereas little deformation was observed in the old cell wall. Toole *et al.* (2001, 2002) analyzed the stiffness (Young's modulus) and strength (breaking force) of cell walls isolated from *Chara*. Hot water treatment increased the stiffness of the cell walls, whereas extraction of pectic polysaccharides with a Ca^{2+}-chelating agent and KOH decreased both the stiffness and strength of the cell walls. These results imply that matrix components may regulate the mechanical properties of the cell wall.

More recently, Proseus and Boyer (2006b) found that exogenous pectin enhances the growth rate of living cells in *Chara*, and suggested that loosening of the isolated cell wall occurs because pectin sequesters Ca^{2+} in the cell wall, inhibiting gel matrix formation.

Cell wall extensibility has also been examined in the tip-growing alga *Vaucheria* by measuring the deformation of the apical cell wall fragment caused by multiaxial stress (Mine and Okuda, 2003). When stress is applied to the apical cell walls of *Vaucheria*, they creep first and eventually rupture. However, no significant extension after the application of stress occurs in the cylindrical, basal portion of the cell walls. The extensibility of the cell wall is highest at pH 8.0 but decreases to lower levels under acidic conditions. Pretreatment of the cell wall with a protease considerably weakens cell wall strength, but does not change the pH dependency in the extensibility of the apical cell wall. These results indicate that the mechanical properties of cell walls in *Vaucheria* are distinct from those in higher plants and *Chara*/*Nitella*, which exhibit "acid growth" (Mine and Okuda, 2003). According to observations of fine structures of cell wall surfaces in *Vaucheria* by atomic force microscopy, the amorphous matrix components reside among randomly arranged cellulose microfibrils and the protease treatment removes the amorphous matrix components from the cell wall (Mine and Okuda, 2007). However, in the apical cell wall of the tip-growing region, fine granular matrix components remain among cellulose microfibrils even after protease treatment (Mine and Okuda, 2007). These results imply that amorphous and granular matrix components are involved in maintaining protease-sensitive cell wall strength and pH-dependent cell wall extensibility, respectively (Fig. 2.4). This speculation has been further supported by observations of changes in these matrix components during the branch formation process (Mine *et al.*, 2007), where the strong, inextensible cell wall in the cylindrical region of the cell is transformed to an extensible cell wall in a newly formed growing tip by local blue light illumination (Kataoka, 1975). The internal pressure required to rupture the isolated cell walls, where branch formation is induced by local illumination, decreased remarkably during the early to middle stages of growing tip development (Mine *et al.*, 2007). The wall become extensible by internal pressure during the middle and late stages, indicating that the wall was weakened first and then the wall extensibility was increased (Mine *et al.*, 2007). The fine granular matrix components appeared in patches during the early and middle stages of the branch formation, implying the involvement of the wall components in rendering the cell wall extensible (Mine *et al.*, 2007).

4.1.4. Signal transduction and hormonal control

Although not studied exhaustively, external signals may interfere with morphogenesis in giant-celled algae. In addition to the effect of temperature and light quality (see above), Ca^{2+} has been shown to influence whorl

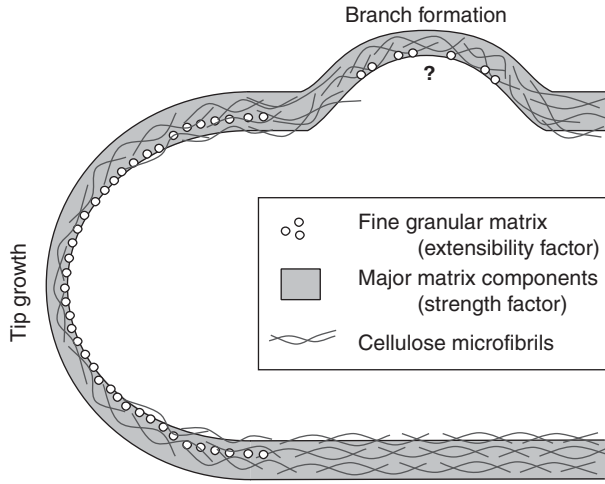

Figure 2.4 Cell wall architecture during vegetative development in *Vaucheria*, deduced from cell wall extension experiments and fine structural observations of cell wall surfaces (Mine and Okuda, 2003, 2007). A cell wall consists mainly of cellulose microfibrils and major matrix components, which embed the microfibrils and are concerned with the maintenance of cell wall strength as a strength factor. The cylindrical basal region of the cell wall is mechanically stronger and less extensible than the cell wall in the apical region where tip growth of the cell occurs. In the tip-growing region, a fine granular matrix is present among microfibrils and promotes pH-dependent extensibility of the cell wall as the extensibility factor. On branch formation in the cylindrical, nongrowing region, reduction of cell wall strength and promotion of its extensibility might occur by alternation of activities of these factors for cell wall mechanical properties.

morphogenesis in *Acetabularia* (Harrison *et al.*, 1988) and might act by signaling to the cytoskeleton (Goodwin and Briere, 1994). Above all, wound-healing responses have been the subject of studies on cell signal transduction in the giant-celled algae, most likely because the rapid, obvious reactions have been suitable for inhibitory experiments. The wound-healing contraction of *Ernodesmis* protoplasm was reversibly inhibited by depleting Ca^{2+} from the external medium and adding the inorganic calcium channel blocker La^{3+} (La Claire, 1982a), indicating that extracellular calcium ions, entering through a calcium channel in the plasma membrane, would be a potential second messenger in the signal transduction chain from wounding to protoplasmic contraction. This was further supported by the moderate inhibition in the permeabilized cell models of trifluoroperazine, a potent inhibitor of calmodulin, in the presence of Ca^{2+} (La Claire, 1984). Similar results have been obtained in the cytoplasmic contraction localized at the cut end in *Acetabularia* (Menzel and Elsner-Menzel, 1989b), and, because calcium ionophore A23187 caused a slow contraction of the whole cytoplasm, it was concluded that the

influx of extracellular Ca^{2+} occurs localized at the wound site, where it is required for the organized contractile reaction that leads to a sealing of the cytoplasmic wound. Protoplasmic aggregation in wounded *Ventricaria* also required external Ca^{2+} in the culture medium (Nawata *et al.*, 1993), and the involvement of calcium-dependent protein kinase was suggested by inhibitory experiments and by biochemical and histochemical identification using a specific antibody (Sugiyama *et al.*, 2000).

Growth of the repair shoot cell produced in the wound-healing reaction in the red alga *Griffithsia* was controlled by a small endogenous glycoprotein, called rhodomorphin (Waaland and Watson, 1980). This growth hormone was further purified and characterized by Watson and Waaland (1986), using a bioassay system in which the growth of a repair cell was stopped, when the endogenous hormone was removed, followed by the resumption of growth when a purified sample of this glycoprotein was added back. This study showed that the molecular mass of the hormone was 15–17.5 kDa and a disulfide bond in the molecule is necessary for biological activity.

4.2. Expression of genes controlling morphogenesis in *Acetabularia*

Transportation of mRNA to and its localization in a specific region, where the gene product is needed, has been regarded as one of the important posttranscriptional controls in gene expression, and the RNAs of a number of genes concerning morphogenesis have been known to be localized to specific regions of the cell, where morphogenetic events occur (Tekotte and Davis, 2002; Wilhelm and Vale, 1993). *Actabularia* is the best material for studying such a phenomenon in giant-celled algae. As reviewed by Hämmerling (1963), early amputation experiments in *Acetabularia* have shown that the apical portion of the cell cut off from the stalk continues to undergo normal vegetative morphogenesis in the absence of the nucleus, whereas the excised middle piece without the apex and without the nucleus does not grow, and, on the basis of these results, accumulation of morphogenetic substances in the apical cytoplasm was postulated (Hämmerling, 1934). After progress in molecular biology had laid the foundation to understand the physical basis of gene expression, such substances have been interpreted as the mRNAs of morphogenesis-related genes. The hypothesis that mRNA is transcribed in the nucleus down in the rhizoid and then transported to the anterior end, where tip growth and apical morphogenesis take places has been further supported by biochemical studies, which demonstrated the intracellular distribution and movement of polyadenylated RNA [poly(A)$^+$ RNA] (Kloppstech and Schweiger, 1975, 1982) and the occurrence of protein synthesis (Lüttke and Grawe, 1984) in nucleate and anucleate cells, all of which agree with earlier histochemical data (Werz, 1960).

A number of physiological, morphological, and biochemical studies related to this morphogenetic mRNA have been conducted and reviewed previously (Berger *et al.*, 1987; Menzel, 1994), and approaches using modern molecular biological technique have been carried out (Henry *et al.*, 2004). Here we address only a few studies related to mRNA distribution in this alga, published after these reviews. Vogel *et al.* (2002) surveyed mRNA that exhibited specific distribution in the vegetative *Acetabularia* cell, using reverse transcriptase-polymerase chain reaction and samples collected along the length of the stalk, and found four mRNA classes that were differentially distributed. The apically localized mRNA pool that is removed by amputation from the anterior end of the cell is thought to be restored by fresh transcript issued from the primary nucleus after amputation and transported via the actin cable system to the regenerating apical end. This assumption has been experimentally substantiated by the fact that restoration of the full morphogenetic capacity of the apex was inhibited by cytochalasin D, and it was further shown that newly transcribed actin-1 mRNA accumulated at the regenerating apex, indicating the involvement of the actin cytoskeleton in transportation and localization of specific mRNAs in this alga (Vogel *et al.*, 2002).

To reveal the occurrence of long-distance transportation of mRNA in the vegetative giant cells of *Acetabularia*, we visualized the distribution of mRNA, as poly(A)$^+$ RNA, by a fluorescence *in situ* hybridization technique using labeled oligo(dT) as a probe (Mine *et al.*, 2001). As a result, accumulation of poly(A)$^+$ RNA was observed in the perinuclear cytoplasm of the rhizoid, and along the stalk in a great number of longitudinal striations of poly(A)$^+$ RNA. Each of these striations consisted of a long tapering tail with a broad head segment typical of the so-called headed streaming bands, which represent the fast long-distance transport mode in this alga (for a summary of this phenomenon see Menzel, 1994). The longitudinal striations of poly(A)$^+$ RNA are closely associated with the thick bundles of actin filaments, which run through the entire length of the cell, and both the striations of poly(A)$^+$ RNA and actin filaments are simultaneously broken down by the treatment with cytochalasin D. These results were considered to represent the morphological evidence of the long-distance transport through the stalk and accumulation of mRNA in the tip-growing region and also imply that the poly(A)$^+$ RNA striations are the mRNA populations that commute between the nucleus and the growing apex of the cell (Fig. 2.5) (Mine *et al.*, 2001). Furthermore, similar observations were conducted on the cells undergoing reproductive development and revealed the disappearance of poly(A)$^+$ RNA striations and the emergence of distinct perinuclear poly(A)$^+$ RNA populations around the haploid nuclei during cyst formation of this alga (Mine *et al.*, 2005).

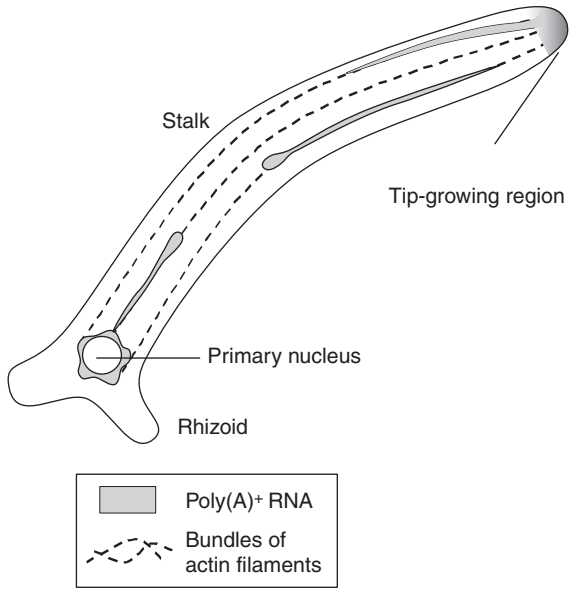

Figure 2.5 **mRNA transportation in vegetative phase of diploid generation of *Acetabularia*. Poly(A)$^+$ RNA accumulates in the tip-growing region of the cell and around the primary nucleus in the rhizoid and is found as long, headed striations that are localized along bundles of actin filaments arranged longitudinally in the cytoplasm of stalk. These striations may represent the intracellular structure concerning the transportation of mRNA of the genes for vegetative morphogenesis of the giant cell from the primary nucleus toward the tip-growing region where most morphogenetic events are occurring.**

5. Concluding Remarks

Possible perspectives and directions toward future research should take into account the two apparently opposite ends of the same line in basic biology: (1) the originality of the phenomenon expressed with a multiplicity of highly diverse features, each typical for just one small cluster of related taxa and (2) the universality of the underlying mechanisms that emerges only after clearing out all the lineage-specific details.

Giant-celled algae may be seen under these two aspects. Notwithstanding the obvious diversity seen in this group of algae, general phenomena such as actin-based long-distance transport and contractile phenomena, membrane dynamics, microtubule-based control of wall microfibrils, shaping processes of the protoplasm, determination of cell polarity, regulation of ionic relations and turgor pressure and, last but not least, aspects of communication between the cell and its environment, can all be studied by choosing one or the other member from this interesting group of organisms.

Despite the advantages that come with the large cell size, there are also certain caveats in the use of these organisms for cell biological studies. The most severe problem, we believe, is the fact that genetic transformation is not yet feasible and selection of mutants is often difficult with possibly the rare exception of *Acetabularia*, which also turned out to be a good experimental model for the visualization of gene expression in the course of development. As far as other members of the giant-celled algae are concerned, physical and chemical manipulation still holds considerable promise for progress in the understanding of fundamental biological processes, especially those concerning ion influx/efflux and cell wall extensibility, before methods for genetic manipulation will become available on a larger scale for this unique group of organisms.

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CHAPTER THREE

Plasmodium in the Postgenomic Era: New Insights into the Molecular Cell Biology of Malaria Parasites

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Abstract

In this review, we bring together some of the approaches toward understanding the cellular and molecular biology of *Plasmodium* species and their interaction with their host red blood cells. Considerable impetus has come from the development of new methods of molecular genetics and bioinformatics, and it is important to evaluate the wealth of these novel data in the context of basic cell biology. We describe how these approaches are gaining valuable insights into the parasite–host cell interaction, including (1) the multistep process of red blood cell invasion by the merozoite; (2) the mechanisms by which the intracellular parasite feeds on the red blood cell and exports parasite proteins to modify its cytoadherent properties; (3) the modulation of the cell cycle by sensing the environmental tryptophan-related molecules; (4) the mechanism used to survive in a low Ca^{2+} concentration inside red blood cells; (5) the activation of signal transduction machinery and the regulation of intracellular calcium; (6) transfection technology; and (7) transcriptional regulation and genome-wide mRNA studies in *Plasmodium falciparum*.

Key Words: Malaria, Merozoite invasion, Ca^{2+} signaling, *Plasmodium* transfection, Transcriptional regulation, Gene expression.

1. Introduction

The genus *Plasmodium* is composed of unicellular eukaryotes of the phylum Apicomplexa that invade and parasitize red blood cells of terrestrial vertebrates as part of their complex life cycles. Included species are the major human malaria pathogens *Plasmodium falciparum* and *Plasmodium vivax*, both of which are presently the subjects of renewed global research aimed at identifying targets for their prophylaxis and treatment. Malaria parasites are highly successful at exploiting their intracellular habitat, and it is increasingly

clear that their survival strategies are highly complex and subtle, requiring equally subtle science to study them (Aravind *et al.*, 2003; Kuhn *et al.*, 2007; Rosenthal, 2004; Soldati *et al.*, 2004; Templeton and Deitsch, 2005).

The genus *Plasmodium* contains many species some of whose genomes have been either fully or partially sequenced. However, deciphering and validating gene function and the mechanisms controlling gene expression require molecular and cellular techniques that extend beyond genome sequence alone. The new methods (Abraham *et al.*, 2004; Kappe *et al.*, 2001; Khan *et al.*, 2005; Mair *et al.*, 2006; Silvestrini *et al.*, 2005) are also opening up new areas of research into the regulation of gene transcription and protein synthesis with particular advances in gene modification and bioinformatics demonstrating unique features of gene expression in *Plasmodium* that emphasize the research challenges this genus continues to provide.

Setting up the data on structure of *Plasmodium* organelles, transfection, cellular and molecular physiology, and bioinformatics will help us to understand questions such as gene and transcriptional regulation, how proteins interact with each other to perform a task, and how and when a specific second messenger is put in action to control the complex malarial cell cycle.

2. Cellular and Molecular Biology of Red Blood Cell Invasion

Malaria parasites, like most apicomplexans, exploit a highly specialized parasitological niche within their hosts, namely the intracellular habitat, enabling them to grow and multiply with minimal surveillance by the host's immune system, in a nutrient-rich environment. A crucial factor in exploiting this resource is the ability of the parasites to enter, feed on, and multiply within their host cells without lysing them. The diminutive size of the parasite and the optical limits of light microscopy demand the much higher resolution of electron microscopy to observe the cellular events of invasion in detail. Ladda and coworkers (1969) published the first ultrastructural account of merozoite invasion into the red cell (RBC) and established that the malaria merozoite enters it not by piercing its membrane but by causing it to form a deep invagination that eventually encloses the parasite completely in an intracellular compartment (the parasitophorous vacuole, PV) lined by a membrane (parasitophorous vacuole membrane, PVM) within the RBC (Fig. 3.1A and B). Subsequent electron microscopy studies confirmed this scenario and added further details (Bannister and Dluzewski, 1990; Galinski *et al.*, 2005). Molecular mechanisms underlying invasion are currently under intense investigation, although much of the data have yet to be satisfactorily correlated with ultrastructural observations (Cowman and Crabb, 2006; Galinski *et al.*, 2005). In this account, the

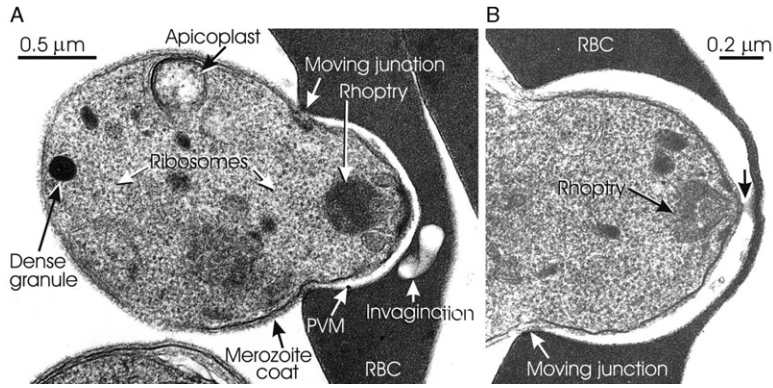

Figure 3.1 (A and B) Electron micrographs showing merozoites of *Plasmodium knowlesi* in the process of RBC invasion. In (A) the merozoite has begun to move into the deepening invasion pit lined by the developing parasitophorous vacuole membrane (PVM), the thick merozoite coat being removed at the moving junction; a small membrane-line invagination of the PVM extending deeper into the RBC is also visible. In (B), the anterior end of an invading merozoite is shown at a higher magnification, illustrating the connection between the rhoptry exit and the PVM (arrow).

available evidence is briefly surveyed and some outstanding questions are identified. To set the scene, the formation and structure of the merozoite are briefly described, followed by a consideration of how merozoites escape from the infected RBC (IRBC) and then invade a fresh one.

2.1. Structure of merozoites

Although amongst the smallest of eukaryotic cells, merozoites carry a full complement of equipment needed to invade and begin exploiting the RBCs of their host. Invasional structures include a selectively adhesive coat, three sets of secretory organelles (micronemes, rhoptries, and dense granules), a minimal cytoskeleton (microtubules, polar rings, etc.) and, attached beneath the plasma membrane, a flat cisterna (inner membrane complex, IMC) important for motility. A small nucleus, a mitochondrion, and an apicoplast carry with them the genetic and metabolic apparatus needed for the following phases of the life cycle. Trafficking organelles such a rough endoplasmic reticulum (RER) and Golgi complex are absent or residual in the mature merozoite, although the cytoplasm is packed with unattached ribosomes, indicating that all unnecessary cellular activities are halted until a new RBC is entered.

When first released from the schizont, merozoites are ellipsoidal and range in length from 1.2 µm in *P. falciparum* (Langreth *et al.*, 1978) to 3.5 µm in the avian parasite *P. fallax* (Aikawa, 1967). A flat-topped

mound (the apical prominence) protrudes at its anterior (apical) end, where the three cytoskeletal annuli, the polar rings, are located. At the other end of the merozoite is a small bump marking the point of separation from the parent schizont mass (Bannister and Dluzewski, 1990; Bannister *et al.*, 2000a).

2.1.1. Merozoite surface

The merozoite surface is covered completely by a thick (15 nm) coat of narrow upright filaments each about 4 nm thick with wider tips, usually grouped in small clumps (Bannister and Dluzewski, 1990; Bannister *et al.*, 1986b). Genomic analysis has identified 20 or so proteins associated with the parasite surface coat before exit (Cowman and Crabb, 2006). Nine of these (core proteins) are anchored with glycosylphosphatidylinositol (GPI) to the plasma membrane, whereas the others (peripheral proteins) are attached to the GPI-anchored proteins. The most abundant core protein is merozoite surface protein-1 (MSP-1) (Holder, 1994; Holder *et al.*, 1992) synthesized (in *P. falciparum*) as a 195-kDa GPI-anchored molecule in the endoplasmic reticulum (ER) and processed in the schizont to a complex of four fragments that remain together noncovalently on the free merozoite surface until invasion (Blackman and Holder, 1992). The GPI-anchored fragment, MSP-1_{42}, is cleaved again at invasion to leave only a 19-kDa portion (MSP-1_{19}) still attached to the merozoite as it enters the RBC. Two peripheral proteins, MSP-6 and MSP-7, are also associated with MSP-1 noncovalently and are shed with the large fragments of MSP-1 during invasion (Kauth *et al.*, 2006). Other MSPs such as MSP-9 (ABRA; acidic–basic repeat antigen) are only loosely attached (Vargas-Serrato *et al.*, 2002).

A third set of surface proteins comprises those secreted by micronemes. Most of these are transmembrane proteins that reach the merozoite surface only after exit from the schizont-infected RBC. They are discussed later in relation to invasion. Finally, a rather neglected set of surface-accessible molecules includes intrinsic membrane proteins such as ion channels, signaling complexes, and cytoskeletal attachment sites. Some of these are visualized in electron microscopic freeze–fracture images showing numerous intramembranous particles (IMPs) (Aikawa *et al.*, 1981; McLaren *et al.*, 1979), reminding us of an important class of molecules that have yet to be explored.

2.1.2. Merozoite pellicle and cytoskeleton

Like motile stages of many other apicomplexans, the merozoite surface is formed by three membranes, collectively termed the pellicle. This consists of the plasma membrane and, close under it, the two membranes of the inner membrane complex (IMC) forming a closed flat cisterna. The anterior edge of the IMC is attached to the outer surface of the third (most posterior) polar ring and elsewhere it is connected to the plasma membrane by

numerous thin filamentous links (Bannister and Mitchell, 1995). A few subpellicular microtubules are attached to the undersurface of the IMC by short side filaments.

The numbers and arrangement of subpellicular microtubules vary with species. In *P. falciparum*, there are only two or sometimes three, and running longitudinally in parallel along the side of the merozoite between the third polar ring anteriorly and mid-nuclear level posteriorly (an arrangement termed the *falciparum* merozoite assemblage of subpellicular microtubules, or *f*-MAST; Bannister *et al.*, 2000a, 2003). The microtubule ''plus'' ends are at the polar ring attachment where the microtubule-initiating protein γ-tubulin has been localized by immunofluorescence microscopy (IFA) (Fowler *et al.*, 2001). In other species they may be more numerous, for example, *P. fallax* merozoites, in which eight or more are grouped together (Aikawa, 1967), a feature perhaps related to their greater size and need for shape maintenance. Besides such a likely mechanical role, subpellicular microtubules are important in microneme targeting during merozoite assembly (Bannister *et al.*, 2003) (see below).

The three polar rings at the anterior (apical) end of the merozoite form a cytoskeletal support for the apical prominence, and define the site of rhoptry and microneme secretion during invasion, and the location of apical junction formation with the RBC (see below). Because the third polar ring (the most posterior and largest) forms the anterior attachment for the IMC externally and the subpellicular microtubules internally, it is clearly a highly significant element of merozoite organization. Another cytoskeletal feature of the merozoite is the cytostome, a dense annulus 150–200 nm in diameter lying under the plasma membrane on one side of the parasite in a small gap in the IMC. After invasion this structure is used for feeding on RBC cytoplasm (Aikawa *et al.*, 1967), but it is inactive in the merozoite stage.

2.1.3. Secretory organelles

Micronemes, rhoptries, and dense granules are clustered in the anterior half of the cell. Micronemes are numerous (20 or more per merozoite in *P. falciparum*), elongate, densely staining vesicles clustered apically. Their shapes and sizes vary with the species. In *P. falciparum*, they look like long-necked bottles about 110 nm long and 65 nm at their widest (Bannister *et al.*, 2003, 2005) but in others, for example, *P. knowlesi*, they are thinner, curved banana-like vesicles. They are grouped on either side of the rhoptries, and in immunofluorescence antibody (IFA) imaging often appear as two lobes that can be mistaken for those organelles.

Proteins shown by IEM to be micronemal or suspected of being so on the basis of their IFA localization include several groups of adhesins and one or more proteases (see below). Micronemes may consist of more than one functional population, as apical membrane antigen-1 (AMA-1) and erythrocyte-binding antigen-175 (EBA-175) do not exactly colocalize in

IFA preparations (Bannister *et al.*, 2003; Healer *et al.*, 2002), so sequential secretion of different proteins in different phases of invasion is a possibility.

Rhoptries are much larger than micronemes and are only two in number. They are pear shaped, with their pointed ends converging on the center of the apical prominence (Bannister *et al.*, 2000b). They appear to be mainly or entirely responsible for generating the parasitophorous vacuole during invasion (see below). Although there is a single enclosing membrane, two distinct domains with different protein profiles are present, the basal bulbous part containing low molecular weight rhoptry-associated proteins RAP-1, RAP-2, and RAP-3, and the high molecular weight RhopH complex including CLAG9 (where CLAG stands for cytoadherence linked asexual gene), MAEBL (merozoite-adhesive erythrocyte-binding ligand), and RAMA (rhoptry-associated membrane antigen), whereas in the neck region a 225-kDa protein (rhoptry neck protein RON4) is present (Kats *et al.*, 2006; Lebrun *et al.*, 2005; Roger *et al.*, 1988). The GPI-anchored protein RAMA is associated with the rhoptry membrane, and may be important in the trafficking of other rhoptry proteins to these organelles (Topolska *et al.*, 2004a–c). This dual distribution suggests that during invasion the apical and basal proteins have different, sequential functions. How many of these proteins are vital to invasion is uncertain, because this process is not affected by knockouts of some major proteins such as RAP-1.

Dense granules are small spheroidal or ellipsoidal vesicles about 85 nm in diameter (in *P. falciparum*), scattered in the apical half of the merozoite. Only three proteins have been localized to them, ring-infected erythrocyte surface antigen (RESA), ring membrane antigen (RIMA), and possibly subtilisin-like protease-1 (SUB-1), unlike their counterparts in *Toxoplasma gondii*, where several proteins have been identified (Topolska *et al.*, 2004a,c; X. W. Zhou *et al.*, 2005). In *Plasmodium* merozoites, dense granules discharge their contents by exocytosis at the merozoite surface after invasion (see below).

2.1.4. Other organelles

Each merozoite also contains a single mitochondrion with small numbers of tubular cristae and a single apicoplast with a triple membrane wall (Hopkins *et al.*, 1999). In *P. falciparum*, these two organelles lie side by side along the flank of the merozoite, with the apicoplast attached to the subpellicular microtubule doublet/triplet (Bannister *et al.*, 2003; Hopkins *et al.*, 1999; Tonkin *et al.*, 2006a). In addition, there are numerous free ribosomes in the monosomal configuration (i.e., not in polysome clusters and therefore, in the free merozoite, not engaged in translation). In the nucleus, which lies posteriorly in the merozoite, the chromatin configuration differs between species: in *P. falciparum* it is completely euchromatic (uniformly finely fibrillar) whereas for example in the larger *P. fallax*, dense clumps of heterochromatin

are present around its periphery (Aikawa, 1966). No nucleoli are present—a surprising finding surprising finding yet to be explained.

2.2. Merozoite assembly

Proteomic analysis shows that immediately before and during the schizont stage there is a period of intense protein synthesis in which the specific merozoite proteins are produced (Bozdech *et al.*, 2003; Hall *et al.*, 2005). This gradually tails off until it ends just before merozoites exit from the parasitized red blood cell. During the schizont stage, the genome undergoes a series of replications interspersed by endomitotic nuclear divisions in which mitotic spindles separate the chromosomes into dividing nuclei without nuclear membrane lysis. Spindle pole bodies (SPBs) are located in nuclear pores. In *P. falciparum*, there are four rounds of alternating DNA replication and mitosis in about 4 h (White and Kilbey, 1996). Half-way through the schizont stage, merozoites begin to bud from the surface of the parasite, each bud formed opposite an SPB of the final mitosis (Aikawa, 1966). Each bud receives a nucleus followed by a mitochondrion and an apicoplast. As the bud elongates, the inner membrane complex is assembled beneath the plasma membrane, and subpellicular microtubules are formed at the polar rings, which also appear at this time (Bannister *et al.*, 2000a). A Golgi complex consisting of one or two cisternae lies close to the nucleus, and receives coated vesicles from the apical surface of the nuclear envelope, close to the SPB (Bannister *et al.*, 2000a; Langreth *et al.*, 1978). The RER is a branched convoluted cisterna confluent with the nuclear envelope, which also bears ribosomes. This trafficking pathway (ER—nuclear envelope—Golgi complex) is also seen in *Toxoplasma* (Hager *et al.*, 1999) and is probably typical of the Apicomplexa. Free ribosomes are also abundant in the cytosol of the developing bud, indicating extensive synthesis of cytosolic proteins including those of the cytoskeleton.

The formation of rhoptries, micronemes, and dense granules has been studied in greatest detail in *P. falciparum*. They are generated in sequence close to the Golgi complex, rhoptries appearing first early in schizogony at the end of the second mitosis, as small paired spherical vesicles close to the SPBs (Jaikaria *et al.*, 1993; Margos *et al.*, 2004). They enlarge steadily by fusion of small vesicles from neighboring Golgi cisternae, eventually transforming to the characteristic pear-like appearance in the mature merozoite (Bannister *et al.*, 2000b; Jaikaria *et al.*, 1993). Micronemes begin to be generated late in the schizont period, in merozoite buds after the last mitosis. After their formation at the Golgi complex, they become attached to the subpellicular microtubules by narrow filaments radiating from the microneme surface, then move apically along the microtubules to their docking zone with the apical membrane (Bannister *et al.*, 2003). The microtubule-associated motor proteins dynein(s) and kinesin(s) are abundant in the apical

part of the merozoite (Fowler *et al.*, 2001, 2004) and either could be the means of microneme propulsion. Finally, in the nearly mature bud, dense granule formation begins, again at the Golgi complex, these organelles passing out into the apical cytoplasm apparently at random. The production of secretory organelles ceases as a ring of constriction at the base of the merozoite bud pinches it off from the residual body of the schizont, and the Golgi complex and RER disappear before merozoites are released from the IRBC.

2.3. Merozoite exit from infected red blood cells

To reinvade, merozoites must first escape from their place of birth through two enclosing membranes—the PVM and the IRBC membrane. Merozoite emergence is a rapid event, described initially by Winograd *et al.* (1999) as emergence through a small aperture in the RBC membrane, suggesting a fusion of the RBC and PV membranes at this point. However, treatment of schizonts with the cysteine protease inhibitor E64 allows the RBC to lyse first, suggesting that this might be the normal sequence (Salmon *et al.*, 2001). This conclusion was in turn countered by Wickham and colleagues (2003), who found that labeled chimeric proteins introduced into the RBC cytosol flooded the PV before merozoite exit, indicating that the PVM was the first to be breached. More recently, work by Glushakova and coworkers (2005), using low-light imaging of fluorescent quantum dot-labeled IRBC and PV membranes, concluded that the two membranes lyse simultaneously although separately, probably as the result of a sudden osmotically driven swelling within the PV. The trigger for this event has yet to be defined, but crucially, because exit can be prevented with a range of inhibitors, protease activity must form at least part of the exit mechanism (Hadley *et al.*, 1983; Salmon *et al.*, 2001). Recently it has been shown that the protease PfSUB-1 is released from a novel type of merozoite vesicle (exoneme) into the PV just prior to schizont rupture to cleave various PV proteins including SERA 5 (serine-rich antigen 5), which may initiate more extensive proteolytic activity leading to breakdown of the PVM and RBC membrane (Blackman *et al.*, 2004; Yeoh *et al.*, 2007).

2.4. Invasion

Visual details of this complex process come mainly from recorded images of live *P. knowlesi* in culture (Dvorak *et al.*, 1975) and electron microscopy of fixed samples (Bannister and Dluzewski, 1990). It can be divided into seven stages: (1) initial adhesion, (2) contact between the merozoite apex and the RBC, leading to apical junction formation, (3) generation of the PVM, overlapping with (4) movement of the merozoite into the PV, (5) the sealing of the merozoite into the PV, (6) discharge of dense granules into the PV, and (7) merozoite transformation into the ring stage.

2.4.1. Initial adhesion

When a merozoite contacts an appropriate RBC it can adhere to it by any part of the parasite's surface. This is followed by the merozoite rolling around on the RBC surface, accompanied by heaving movements of the RBC, which becomes partially wrapped around the merozoite. This type of contact may be only temporary, the merozoite often detaching and adhering elsewhere to the RBC or to another. The movements are not inhibited by cytochalasins (Miller *et al.*, 1979), and are likely to be related to the dynamics of brief receptor–ligand interactions rather than merozoite motility. The thick merozoite coat is responsible for this form of adhesion, at a membrane-to-membrane distance of 20–30 nm, bridged by merozoite coat filaments.

2.4.2. Apical junction formation

When the merozoite apex contacts the RBC surface, a more permanent interaction occurs to form the apical junction, with special structural features on both RBC and merozoite sides of the apposition. The membrane of the merozoite's apical prominence approaches the RBC surface to about 10 nm, thin filaments eventually crossing the gap (Aikawa *et al.*, 1978). Beneath the RBC membrane, a layer of densely staining material, presumably RBC membrane cytoskeleton components, cluster. This appears to be an irreversible step, and is necessary for invasion to proceed. Later, the junction transforms into a motile structure, termed the moving junction (see below).

2.4.3. Formation of parasitophorous vacuoles

Once the apical junction has been established, one or more small local invaginations appear in the RBC surface, often leading deep into the RBC interior. One of these grows into a much larger indentation into which the merozoite glides before being sealed within the RBC (Fig. 3.1A and B). Whether this represents a simple in-tucking of the RBC surface or the generation of new membrane by the parasite is still uncertain, the conflicting evidence suggesting a combination of these two processes (see Dluzewski *et al.*, 1992, 1995; Galinski *et al.*, 2005 for review). Whatever the mechanism, it is clear that the RBC membrane is profoundly altered at invasion, and that secretion from the merozoite rhoptries is largely if not wholly responsible for the changes. Most striking is the removal of many membrane proteins and probably all the attached membrane skeleton from the invasion pit and its successor, the early PV (Aikawa *et al.*, 1979; Atkinson *et al.*, 1988; Dluzewski *et al.*, 1989) indicating that proteases are released at the point of apical contact with the RBC. Interestingly, a few minor RBC membranes do enter the PV including flotillins-1 and -2, aquaporin-1, and scramblase (Murphy *et al.*, 2004), all of them lipid raft proteins. A number of lipid

raft-associated parasite proteins of rhoptry origin (e.g., Pf RhopH2 and Pf stomatin) also enter the vacuole, suggesting the importance of lipid rafts in PVM formation (Hiller *et al.*, 2003).

Electron microscopy has shown that as the merozoite moves into the invasion pit, its apex retains contact with the invaginating RBC surface via a column of membrane-like material emanating from the common aperture of the rhoptries in the center of the apical prominence. This appears to be continuous with the pit membrane, suggesting that rhoptry contents are flowing into the membrane to cause its expansion (Bannister and Mitchell, 1989). Multilamellar material also exits from the common rhoptry duct if the merozoite detaches (Bannister *et al.*, 1986a), or if prevented from entering the RBC after cytochalasin treatment (Dluzewski *et al.*, 1989). Proteomic studies have not yet thrown much light on the mechanism of these events, which are likely to require the coordinated function of several or many molecular entities including proteases, lipases, membrane fusion proteins, and lipids.

2.4.4. Parasite entry into red blood cells: moving junction and invasion motor

Inhibitors of actin–myosin-based motility allow the formation of a close apical junction, but prevent the movement of the parasite into the abortively enlarging invasion pit (Dluzewski *et al.*, 1989; Miller *et al.*, 1979). Normally the junction expands into a ring of contact with the rim of the pit (the moving junction), which tracks back over the merozoite surface (Aikawa *et al.*, 1978) as the merozoite progresses forward, a process taking less than 1 min to complete (Dvorak *et al.*, 1975). This movement resembles the much more extensive gliding motility seen in *Plasmodium* sporozoites and invasional stages of other apicomplexans, investigated most fully in *Toxoplasma* (Cowman and Crabb, 2006; Heintzelman, 2006; Kappe *et al.*, 2004; Opitz and Soldati, 2002; Sibley, 2004). There is much evidence that the motive force is a sliding interaction between myosin molecules attached to the parasite's inner membrane complex and filamentous actin linked by accessory proteins through the plasma membrane to the RBC surface. Although the microscopic structure of the invasion motor has not yet been described, several key molecules of the motor have been identified and a theoretical model has been proposed.

Plasmodium actin G-actin monomer is abundant in merozoites, and can be polymerized under appropriate conditions to form F-actin restricted in length to about 100 nm (Schmitz *et al.*, 2005). Actin-capping proteins have also been isolated, and it has been suggested that to initiate the formation of the invasion motor, these have to be removed to allow the growth of filaments to lengths able to support myosin force generation (Schuler and Matuschewski, 2006; Baum *et al.*, 2006a). Presumably apical contact by the

merozoite is needed to start the assembly of the motor in the apical prominence, but how it is switched on and later, off, is yet to be explored.

The other major component of the invasion motor is a class XIV myosin attached to a protein complex that includes a connecting protein (MTIP; myosin A tail domain-interacting protein) attached to membrane anchors [gliding-associated protein (GAP)-45, GAP-50] (Baum *et al.*, 2006b; Bergman *et al.*, 2003; Gaskins *et al.*, 2004; Pinder *et al.*, 1998). From molecular and IEM data, it is envisaged that actin filaments are assembled just beneath the plasma membrane at the apical/moving junction, attached to aldolase, which in turn is linked to a transmembrane protein, possibly merozoite thrombospondin-related adhesive protein (MTRAP), with other secreted proteins such as EBA-175, AMA-1, and the rhoptry neck protein PfRON4 (Alexander *et al.*, 2005) participating. On the other side of the junction, RBC surface components including (in *P. vivax*) the Duffy antigen and in *P. falciparum* sialylated glycoproteins such as glycophorin A are likely to be ligands (Galinski *et al.*, 2005; Cowman and Crabb, 2006; see also below). Directional cycling of myosin–actin cross-links would then propel the junction posterior along the merozoite pellicle, with consequent anterior movement of the merozoite into the invasion pit. Apart from the Duffy–DBP link, these receptors and ligands at present lack direct evidence for motor participation, and it is likely that the final picture will be rather different from the present one. How the motor is assembled, what switches it on and off, what determines its direction and rate, and how the force is transmitted to the whole merozoite (which in the case of *P. falciparum* has only a minimal array of microtubules) are some of the questions yet to be addressed.

2.4.5. Late invasional events

Invasion ends with a sequence of further changes. First, the PV is sealed off by the fusion of the RBC membrane across the mouth of the pit, and the PVM also seals and detaches from the RBC surface. Second, the dense granules move to the merozoite surface and fuse with it to release their contents into the PV, causing the further expansion of the PVM (Aikawa *et al.*, 1990; Bannister *et al.*, 1975). At least one of the secreted proteins, RESA, then crosses the PVM and passes into the RBC cytosol, where it reaches the RBC membrane skeleton and binds to spectrin in the RBC membrane skeleton (Foley *et al.*, 1991). This interaction increases the thermal stability of the RBC (Silva *et al.*, 2005), which could be important to the parasite under fever conditions. However, evidence indicates that the RESA–spectrin interaction blocks later invasion into the infected RBC by other merozoites, clearly an important survival strategy (Pei *et al.*, 2007).

Finally, the special invasion apparatus of the merozoite disappears as it transforms into the discoidal ring stage. This entails the detachment of the IMC from the surface and its demolition, and the final removal of all traces

of some surface molecules such as the cleaved transmembrane/intracellular domains of AMA-1. The rhomboid proteases (ROMs), which are in *Toxoplasma* involved in cleavage of secreted surface proteins, may be important in the cleansing of invasion transmembrane proteins from the exterior of the parasite by cleaving them within its plasma membrane (Baker *et al.*, 2006; see also below).

2.4.6. Adhesive interactions

There is a considerable literature on the topic of adhesive interactions (Cowman and Crabb, 2006; Galinski *et al.*, 2005), which is steadily lengthening the list of putative adhesins. For technical reasons, most studies have been unable to distinguish between the stages of initial attachment and apical junction formation. It is only when experiments have involved visual observation of invasion that ligands and receptors have been clearly assigned. Adding to this problem, chemical binding assays of isolated proteins do not necessarily reflect their *in vivo* properties, where their precise locations, stage-dependent processing changes, and cellular environment may be crucial to adhesion. Finally, the existence of many variant pathways and abundant functional redundancy has made it difficult to dissect cell invasion processes by genetic or chemical modification experiments.

Putative adhesive molecules include two distinct categories: merozoite coat proteins and those secreted from the apical organelles. Most isolated GPI-anchored coat proteins are able to adhere to RBC membranes, and it seems certain that one or more of them are largely responsible for initial attachment. The best candidate responsible for this initial attachment at present is the dominant coat protein MSP-1. As described above, this protein is cleaved and most of it shed from the merozoite surface at invasion, together with the noncovalently attached peripheral coat proteins MSP-6 and MSP-7 (Pachebat *et al.*, 2001). Antibodies preventing MSP-1 cleavage also inhibit invasion (Blackman *et al.*, 1994). The secreted adhesins (and putative ones inferred from proteomics) represent another battery of adhesive molecules. These proteins possess transmembrane domains and are stored and secreted mainly by micronemes. Included are four classes, distinguishable by their sequence homologies: (1) the Duffy binding-like proteins (DBLs) or erythrocyte binding-like proteins (EBLs); (2) reticulocyte binding-like proteins (RBLs); (3) the thrombospondin-related anonymous protein (TRAP) family; and (4) apical membrane antigen-1 (AMA-1).

The DBLs/EBLs are micronemal proteins capable of binding to RBCs (the only exception is MAEBL, which is a rhoptry protein; Blair *et al.*, 2002). These proteins are present in all *Plasmodium* species, with homologs existing in several other apicomplexan genera as well (Cowman and Crabb, 2006; Galinski *et al.*, 2005). The best understood is the Duffy binding protein (DBP). It has been shown that people of West African origin who

are Duffy negative are protected against *P. vivax* (and, fortunately for research, *P. knowlesi*), but not *P. falciparum* infection.

When human Duffy-negative RBCs are incubated with *P. knowlesi* merozoites, apical junction formation is prevented, although initial adhesion events appear to remain unaffected (Miller *et al.*, 1979). A *P. knowlesi dbl* gene knockout likewise blocks apical junction formation with normal (Duffy-positive) RBCs (Singh *et al.*, 2005). The Duffy antigen (or Duffy-associated chemokine receptor, DARC) is a glycoprotein that binds merozoite-localized DBP with high affinity (Chitnis and Blackman, 2000; Hans *et al.*, 2005) in a manner that is not dependent on its sialic acid residues. The nature of this interaction is not completely clear, however, as Duffy-negative RBCs can still be invaded if treated with trypsin. Adhesins of the DBL family also include a range of secreted proteins in other *Plasmodium* species that bind specifically to various glycophorins at the RBC surface via their terminal sialic acid residues. In *P. falciparum* they comprise the micronemal proteins EBA-175, BAEBL, and JESEBL, the rhoptry protein MAEBL, and EBL-1. Most of these bind only one or two glycophorins. For example, EBA-175 selects glycophorin A and not B, C, or D and fails to invade En(a$^-$) RBCs deficient in glycophorin A (Dolan *et al.*, 1994; Sim *et al.*, 1994). However, a point mutation of EBA-175 has been shown to bind preferentially to glycophorin C (Mayer *et al.*, 2006), thus demonstrating that caution must be exercised when assigning receptor–ligand interactions (see also below).

The RBL family includes several proteins that also bind to sialic acid residues of RBC surface glycoproteins. In some species, RBLs may be responsible for selecting reticulocytes for invasion, for example, by *P. vivax* merozoites. In *P. falciparum*, three expressed RBL genes have been identified and because *P. falciparum* invades RBCs of all ages, in this case the term normocyte-binding proteins (NBPs 1, 2a, 2b, and 3) or reticulocyte-binding-like homologs (Rh1–4) is more appropriate. In *P. yoelii*, members of the large Py235 family of rhoptry genes (at least 14 paralogs) are also able to bind mature RBCs (Ogun and Holder, 1996).

The TRAP family contains several micronemal adhesins expressed in various stages of the parasite life cycle including sporozoites (TRAP, CTRP, MTRAP, and PFF0800). The most significant for merozoite invasion is MTRAP, a transmembrane protein that is able to bind to aldolase and therefore serve as a potential RBC ligand for the moving junction (Thompson *et al.*, 2004).

AMA-1 is a family of proteins present in several apicomplexan genera, represented by only one homolog in *P. falciparum* (Waters *et al.*, 1990). It is secreted from micronemes after exit from the IRBC (Thomas *et al.*, 1990). Antibodies against this protein block invasion, and knockouts are lethal, indicating its importance to the invasion process. The blocking antibody

prevents apical junction formation, indicating that AMA-1 is important either in the merozoite reorientation process or directly in the formation of the apical junction (Mitchell *et al.*, 2004).

The plethora of adhesins discussed herein is testimony to the complexity of the parasite invasion process and the multiplicity of potential invasion pathways. For instance, individual disruption of all members of the DBL family in *P. falciparum* (EBA-140, EBA-175, and EBA-180) did not affect parasite invasion efficiency of normal erythrocytes, indicating redundancy of these parasite ligands (Duraisingh *et al.*, 2003a; Gilberger *et al.*, 2003; Maier *et al.*, 2003; Reed *et al.*, 2000). However, the invasion process of these knockout lines is significantly different from that of the wild-type parasites when the assay is performed with erythrocytes deficient in certain receptors. Disruption of the RBL genes (NBP, PfRh) produced a similar phenotype, suggesting some redundancy in this family as well (Duraisingh *et al.*, 2003a,b; Stubbs *et al.*, 2005; Triglia *et al.*, 2005). Interestingly, some of these genes could be knocked out in some parasite strains, but not in others, indicating that the redundancy of the ligands, the invasion pathways used, and the ability to switch among different pathways might be specific to each parasite line (Duraisingh *et al.*, 2003b; Gilberger *et al.*, 2003). This complexity is undoubtedly the result of the heavy selective pressures such as mutations in RBC invasion ligands or exposure to antibody attack that are operating on the parasite during this extracellular stage of its life cycle.

The mechanisms involved in the switch of the invasion pathways are still unknown. The transcript profile of the PfRh2b knockout line revealed no upregulation of other ligands, indicating the elements for invasion in alternate pathways are already present (Baum *et al.*, 2005). On the other hand, disruption of EBA175 causes the upregulation of PfRh4 and a switch to a sialic acid-independent pathway (Stubbs *et al.*, 2005). Thus it seems that the molecular mechanisms acting in the switch of invasion pathways vary among different ligands and/or parasite strains. There is also much evidence that the various adhesion families alternatively exploit a range of RBC variant ligands, for example, ligands with different levels of sialylation. This can occur by gene switching within a schizont so that merozoites produced by a single parasite can alternatively express different Py235 genes to enable them to exploit different subsets of RBCs (Khan *et al.*, 2001). The data generated in these studies have important implications on vaccine development, suggesting that an efficient invasion-blocking vaccine must include antigens specific for ligands involved in the multiple invasion pathways. It is expected that with improvement in transfection technology, the generation of multiple and conditional knockout lines may aid in elucidating the exact redundancy in the invasion pathway, thus determining which ligands would be most important for the development of invasion-blocking vaccines.

3. Proteases

Proteases are present in all organisms and play many essential functions, which include digestive catabolism; regulated proteolysis; regulation of the secretory system; and activation/maturation of receptors, cell control factors, and enzymes, including other proteases. In *Plasmodium* species, proteases also participate in specific biological pathways such as digestion of hemoglobin, and egression and invasion of the RBC (Withers-Martinez *et al.*, 2004). On the basis of sequence homology, Wu *et al.* (2003) identified, by database mining, 92 putative proteases in the *P. falciparum* genome belonging to 26 different protease families. Of these, microarray analysis indicated that at least 83 proteases are transcribed in the life cycle and 67 are actively transcribed, the majority in the asexual blood cycle. Only a small proportion of these has been identified experimentally, but examples are known in all of the five major protease groups: the aspartic, cysteine, serine, and threonine proteases, and metalloproteases.

3.1. Invasion-related proteases

Evidence indicates that proteases are important in removal of merozoite surface proteins during and after invasion (O'Donnell and Blackman, 2005). Findings indicate that two serine proteases achieve this in sequence. One is a calcium-sensitive serine protease ("sheddase") responsible for cleaving MSP-1 and AMA-1 external to the plasma membrane of the parasite to leave only short stubs attached to it as the moving junction passes back over the merozoite surface, a strong candidate being the subtilisin-like PfSUB-2, a micronemal protein (Harris *et al.*, 2005). A more radical clearance of most transmembrane invasional proteins such as the DBLs appears to be the role of serine proteases of the Rhomboid group (PfROM1–4) (see Baker *et al.*, 2006; O'Donnell and Blackman, 2005; O'Donnell *et al.*, 2006), an action that may be necessary for the parasite to proceed to the ring stage. In *Toxoplasma*, TgROM-1 is secreted from micronemes, whereas TgROMs 4 and 5 are surface proteins, the latter situated at the posterior end of the zoite, where final clearance of surface proteins occurs as the parasite glides forward (Brossier *et al.*, 2005). It is also likely that other proteases are active in altering the RBC membrane and membrane skeleton during invasion, although it is not clear which of the numerous possible proteases predicted from bioinformatics are responsible (Galinski *et al.*, 2005; O'Donnell and Blackman, 2005).

This short account points to the considerable complexity and high level of molecular organization in the invasion process, and the long haul that will be necessary to dissect the various processes at work in this highly successful although deadly group of organisms.

3.2. Proteases of intracellular stages

After invasion, the parasite begins to feed actively on RBC hemoglobin by endocytosis through the cytostome, a cytoskeletal ring close to the surface of the parasite. Depending on parasite stage and species, endocytosed vesicles carry hemoglobin to one or more food vacuoles where proteases are present. Hemoglobin degradation is probably the protease pathway best studied in malaria parasites. This essential metabolic system, unique to *Plasmodium* species, is responsible for providing amino acids that are important for protein synthesis, energy metabolism, and keeping the osmolarity constant during parasite development inside the RBC. It has been proposed that this process depends on a coordinated action of *Plasmodium* aspartic, cysteine, and metalloproteases and a dipeptidyl aminopeptidase (Ersmark *et al.*, 2006).

Hemoglobin digestion takes place in an acidic organelle, the food vacuole (FV). Although this process occurs in all parasite forms of the erythrocyte cycle, it is most active during the trophozoite stage. The complete order of action by the proteases involved in hemoglobin degradation is not fully understood, but studies either targeting the proteases with GPF and other tags, or disrupting genes of proteases families, have elucidated many aspects of this process.

The initial cleavage of hemoglobin is probably made by a family of aspartic proteases, the plasmepsins (PMI–PMIV). Subsequent hydrolysis is performed by both plasmepsins and some members of a cysteine protease family, the falcipains (FP2, FP2′, and FP3). Oligopeptides of 10–20 residues then become the substrate for the metalloprotease falcilysin (FLN) (Eggleson *et al.*, 1999). Smaller oligopeptides (5–10 residues) are digested by dipeptidyl aminopeptidase 1 (DPAP1) (Klemba *et al.*, 2004b).

The importance of the cysteine and aspartic proteases in hemoglobin degradation has been studied by disrupting members of both families in *P. falciparum* (Table 3.1). Disruption of three cysteine proteases demonstrated that these enzymes also share overlapping functions (Sijwali and Rosenthal, 2004; Sijwali *et al.*, 2004, 2006). However, whereas parasites knocked out for FP-1 and FP-2′ grew normally, the falcipain-2 knockout grew at a reduced rate, with slower hemoglobin hydrolysis. Together, these data point to a major cysteine protease role of falcipain-2 in hemoglobin digestion. Attempts to knock out FP3 failed, suggesting an essential function for this protease in the erythrocyte cycle (Sijwali *et al.*, 2006). Interestingly, parasites knocked out for falcipain-2 were more sensitive to cysteine and aspartic protease inhibitors, demonstrating that members of these two families of proteases act in cooperation to hydrolyze the hemoglobin (Sijwali and Rosenthal, 2004).

The four food vacuole aspartic proteases have been knocked out, including a double knockout of PMI/PMIV and a triple knockout of

Table 3.1 Phenotypes of *Plasmodium* Species Knockout Lines

Gene	Phenotype	Ref.
PbCHT1	Reduction of infectivity in *Anopheles stephensi*	Dessens *et al.*, 2001
rRNA (*S-type*)	Growth retardation of oocysts	van Spaendonk *et al.*, 2001
msp8	No phenotype	Black *et al.*, 2005
Pf PM1	Reduced growth rate in intraerythrocytic stages	Omara-Opyene *et al.*, 2004
Pf PMI	Reduced growth rate in amino acid-limited medium; increased sensitivity to E64 (cysteine protease inhibitor) and decreased sensitivity to pepstatin A (aspartic protease inhibitor)	Liu *et al.*, 2005
Pf PMI/IV	Reduced growth rate in amino acid-limited and normal medium; increased sensitivity to E64 (cysteine protease inhibitor) and decreased sensitivity to pepstatin A (aspartic protease inhibitor)	Liu *et al.*, 2005
Pf PMII	Alteration of mitochondrial morphology	Omara-Opyene *et al.*, 2004
Pf PMII	Reduced growth rate in amino acid-limited and normal medium; increased sensitivity to E64 (cysteine protease inhibitor) and decreased sensitivity to pepstatin A (aspartic protease inhibitor)	Liu *et al.*, 2005
Pf PMIV	Reduced growth rate in intraerythrocytic stages	Omara-Opyene *et al.*, 2004
Pf HAP	Accumulation of vesicles in the digestive vacuole	Omara-Opyene *et al.*, 2004
Pf HAP	Reduced growth rate in amino acid-limited medium; increased sensitivity to E64 (cysteine protease inhibitor) and decreased sensitivity to pepstatin A (aspartic protease inhibitor)	Liu *et al.*, 2005
Pf FP-1	No phenotype	Sijwali *et al.*, 2004

Table 3.1 (*continued*)

Gene	Phenotype	Ref.
Pf FP-2	Decreased cysteine protease activity and hemoglobin hydrolysis, increased sensibility to cysteine (slightly) and aspartic protease inhibitors (strong)	Sijwali and Rosenthal, 2004
Pf FP-2′	No phenotype	Sijwali *et al.*, 2006
Pf FP-2/ PMI/IV	Reduction of parasite growth in normal (slightly) and isoleucine-only amino acid RPMI (more pronounced); increased sensitivity to aspartic protease inhibitor (more pronounced in isoleucine-only than in normal amino acid medium)	Liu *et al.*, 2006
EF-1α	Prolonged G_1 phase and slower growth	Janse *et al.*, 2003
Pf EMP3	Blocking of transference of PfEMP1 to outside of the erythrocyte membrane	Waterkeyn *et al.*, 2000
KAHRP	Disruption of knob formation and reduction cytoadherence to CD36	Crabb *et al.*, 1997
KAHRP	All three repeats of the protein are important for knob formation and cytoadherence	Rug *et al*,. 2006
PbTRAP	Disruption of sporozoite motility and invasion in the salivary gland and liver	Sultan *et al.*, 1997
MAEBL	Disruption of sporozoite invasion in the salivary gland	Kariu *et al.*, 2002
PbIMC1a	Abnormal sporozoite development and decreased invasion	Khater *et al.*, 2004
PyTRAP	Disruption of sporozoite motility and invasion in the salivary gland	Mota *et al.*, 2001
PkCSP	Disruption of sporozoite differentiation in the oocyst	Kocken *et al.*, 2002
PbCSP	Disruption of sporozoite differentiation in the oocyst	Menard *et al.*, 1997

(*continued*)

Table 3.1 (*continued*)

Gene	Phenotype	Ref.
Pbmap-2	Disruption of cytokinesis, axoneme motility, and chromatin condensation in microgametocytes	Tewari *et al.*, 2005
Pfgig	Reduction of gametocyte production	Gardiner *et al.*, 2005
Pfclag9	Reduced adhesion to CD36	Trenholme *et al.*, 2000
Pbs48/45 and *Pfs48/45*	Reduction of male gamete fertility and zygote formation	van Dijk *et al.*, 2001
PfEBA-175	Efficient invasion of neuraminidase-treated erythrocytes; switch to a sialic acid-independent invasion pathway	Reed *et al.*, 2000
PfEBA-181	No phenotype in W2mef knockout strain, but essential to 3D7 strain	Gilberger *et al.*, 2003
PfEBA-140	Disruption of glycophorin C-binding and invasion pathway	Maier *et al.*, 2003
PfEBA-175	Inefficient invasion of erythrocytes treated with chymotrypsin; switch to chymotrypsin-sensitive invasion pathway	Duraisingh *et al.*, 2003
PfRh2a	No phenotype in 3D7 knockout strain, but essential to D10 strain	Duraisingh *et al.*, 2003
PfRh2b	Inefficient invasion of erythrocytes treated with both neuraminidase and trypsin; switch to a chymotrypsin-resistant/sialic acid-dependent invasion pathway	Duraisingh *et al.*, 2003
PfEBA-175	Up regulation of *PfRh4* and switch to a sialic acid-independent invasion pathway	Stubbs *et al.*, 2005
PfRh4	Inability to invade neuraminidase-treated erythrocytes and to switch to a sialic acid-independent invasion pathway	Stubbs *et al.*, 2005

Table 3.1 (*continued*)

Gene	Phenotype	Ref.
PfRh1	More efficient invasion in erythrocytes treated with neuraminidase and trypsin; switch to a sialic acid-independent invasion pathway	Triglia *et al.*, 2005
PfRh3	No phenotype (pseudogene)	Duraisingh *et al.*, 2002
Pf SERA2, 3, 7, and 8	No phenotype	Miller *et al.*, 2002
Pfsbp1	Disruption of PfEMP1 translocation to red blood cell surface	Cooke *et al.*, 2006
Pf EMP3	Truncated protein: disruption of PfEMP1 translocation to red blood cell surface; Null mutant: no phenotype	Waterkeyn *et al.*, 2000

PMI/PMIV/cysteine protease FP-2 (Liu *et al.*, 2005, 2006; Omara-Opyene *et al.*, 2004). Although the knockout line phenotypes demonstrate that none of the aspartic proteases are essential, indicating functional redundancy, reduced growth rates were observed for all knockouts, suggesting the function overlapping may not be absolute and that there must be some advantage for *P. falciparum* to keep its four functional plasmepsins. Moreover, the reduced growth rates of the knockout lines were much more pronounced in medium depleted of all amino acids, except isoleucine (Liu *et al.*, 2006). On the other hand, FP-2 and the triple-knockout parasites cultured in medium supplemented with all amino acids were much more sensitive to aspartic protease inhibitors than wild-type parasites (Liu *et al.*, 2006). These results suggest external amino acid uptake can partially, but not totally, compensate in hemoglobin digestion and that parasites can survive using hemoglobin as the only amino acid source (except for isoleucine).

Compared with the aspartic and cysteine proteases, the metalloprotease falcilysin (FLN) and the dipeptidyl aminopeptidase 1 (DPAP1) have been much less studied. FLN was at first considered only an FV acid protease involved in hemoglobin derivate oligopeptide digestion. Murata and Goldberg (2003) demonstrated that FLN is also localized in another subcellular compartment. They also showed that this protease is active at both pH 5.2 and 7.2 with distinct substrate selectivity, suggesting another role for FLN beside hemoglobin degradation. The second function of FLN has been discovered by Ponpuak and coworkers (2007), who created a green fluorescent protein (GFP)-tagged FLN, to enable its second subcellular

localization to be determined. The enzyme, localized in the apicoplast, was purified and biochemically characterized, showing that it can degrade the transit peptide present in proteins exported to this organelle. Attempts to disrupt this gene failed, suggesting that one or both FLN functions are not performed by other proteases in *P. falciparum*.

The gene encoding DPAP1 has also been GFP tagged, which allowed the demonstration that DPAP1 is localized in the FV (Klemba *et al.*, 2004b). The enzyme was purified and the biochemical characterization demonstrated a dipeptidyl aminopeptidase activity at acid pH, indicating this protease is responsible for degrading hemoglobin-derived oligopeptides of 5–10 residues, releasing dipeptides. The DPAP1 gene could not be knocked out, suggesting this is an essential process not performed by other enzymes.

In conclusion, data coming from the digestive vacuole protease and also DPAP1 experiments suggest that although there is some overlap of function among the cysteine and aspartic proteases, FLN and DPAP1 play essential, nonredundant roles. This implies that intervention strategies based on single-target inhibition are likely to succeed only against the latter two molecules.

4. Protein Trafficking

The compartmentalization of eukaryotic cells and their highly complex organellar organization demand a coordinated system of protein sorting and transport that can place each protein in the appropriate place in the cell. In general, the protein-trafficking system depends on their possession of peptide sequences that act as signals recognized by components of the cellular transport machinery such as receptors, chaperones, and other molecular classes.

During the blood stages, malaria parasites grow inside RBCs lacking cellular machinery for protein synthesis and transport. However, during these stages, parasites carry out an intense program of RBC modification by exporting parasite-derived proteins to the RBC cytosol and cell surface. The parasite also trafficks proteins to parasite-specific destinations including the PV and organelles such as the apicoplast, micronemes, rhoptries, and dense granules. The existence of at least 12 different secretory destinations for *Plasmodium* proteins has been proposed (Tonkin *et al.*, 2006b), some of which are unique to *Plasmodium* species and are further discussed below.

The mechanisms of protein transport in *P. falciparum* are both similar and distinct from those found in other higher eukaryotes. As in other eukaryotes, the conserved N-terminal hydrophobic signal peptide (S) is present and signals for protein import in the endoplasmic reticulum (ER). It has been demonstrated that once in the ER, the default secretory pathway is

transport to the parasite membrane, PV, or PV membrane. This process is brefeldin A (BFA)-sensitive, indicating this secretory route involves passage through the Golgi (Adisa *et al.*, 2003). It has also been also demonstrated, using a GFP chimera, that there are isolated subcompartments in the PV and that, while the tubulovesicular network (TVN) is partially connected to the PV, the Maurer's cleft is not (Adisa *et al.*, 2003). Protein transport from the PV or from the ER to other destinations requires other peptide signals besides S. The construction of GFP chimeras has allowed the identification of some of these peptide sequences necessary for trafficking to parasite organelles and also beyond the PV limits.

4.1. Trafficking to intracellular compartments of parasites

4.1.1. Rhoptries

As previously discussed, these organelles are responsible for secreting proteins involved in invasion and subsequent PV production. Little is known about protein trafficking to these organelles. However, a few rhoptries proteins harbor a C-terminal tyrosine-rich peptide sequence that might be important for BFA-sensitive transport (Tonkin *et al.*, 2006b).

4.1.2. Micronemes

Protein trafficking of the EBL proteins to the micronemes depends on a conserved cysteine-rich region present in the protein ectodomain and on the accurate timing of expression (Treeck *et al.*, 2006).

4.1.3. Dense granules

Dense granules store proteins that are eventually exported to the PV and the RBC cytoplasm. Although no peptide signals have been attributed to dense granule trafficking, Rug and coworkers demonstrated that correct timing of protein expression is essential proper protein targeting (Rug *et al.*, 2004). In this study, the ring-infected erythrocyte surface antigen (RESA) N terminus was fused to GFP and expressed under the control of either the RESA schizont-specific promoter or the heat shock protein 86 (*hsp86*) trophozoite-specific promoter. When under the control of the RESA promoter, the protein is secreted from the dense granules just after invasion into the newly formed PV and then transported across the PVM to the RBC. When expressed under *hsp86* promoter control, RESA is targeted to the PV but not beyond it.

4.1.4. Food vacuole

The enzymes involved in hemoglobin digestion are transported to the food vacuole (FV) by at least two routes. The secretory pathway for the aspartic protease plasmepsin-II (PMII) was mapped with a GFP-tagged protein

(Klemba *et al.*, 2004a). It was demonstrated that the immature form of the enzyme is transported from the ER to the cytostomal vesicle, probably through the Golgi because the transport is BFA sensitive. This process places PMII between the two vesicular membranes. With the fusion of the cytostomal vesicle with FV, the protein is processed, yielding the mature, active form.

DPAP1 is transported to the FV through another route. GFP-tagged DPAP1 seems to accumulate in the PV, suggesting it must first be secreted outside the parasite membrane and then recovered from the PV (Klemba *et al.*, 2004b).

4.1.5. Apicoplast

Most, if not all parasites of the phylum Apicomplexa, harbor an apicoplast derived from an ancient secondary endosymbiosis (McFadden *et al.*, 1996; Kohler *et al.*, 1997). Essential metabolic processes such as fatty acid metabolism and isoprenoid biosynthesis occur in this organelle (Ralph *et al.*, 2004). However, most apicoplast protein-coding genes have been transferred to the nuclear genome, as is the case with most plastids of various phyla. This indicates that the apicoplast proteins are first translated in the cytoplasm of the parasite and then transported to the lumen of the organelle, across its three or four enclosing membranes.

The use of GFP to establish parasite transgenic lines expressing either whole length or truncated apicoplast fusion proteins has revealed many aspects of apicoplast protein transport. Waller and co-workers identified nuclear-encoded apicoplast-targeted proteins in the genomes of *P. falciparum* and *Toxoplasma gondii* (Waller *et al.*, 1998). They showed by IFA and electron microscopy (EM) that two of these proteins localize in the apicoplast of *T. gondii* and that the N-terminal prosequence, containing both secretion signal and the putative transit peptide, is enough to target GFP to the apicoplast. Later on these authors extended their studies further in *P. falciparum* (Waller *et al.*, 2000). They demonstrated that the prosequences of the acyl carrier protein (ACP) gene of *P. falciparum* and *T. gondii* and the same N-terminal region of the β-ketoacyl-ACP synthase III (FabH) gene of *P. falciparum* possess the information needed to target GFP to the apicoplast in *P. falciparum*. In the same study, this apicoplast-targeting prosequence was further dissected. GFP fusions were made with either only the putative signal (S) or the transit (T) peptide. S–GFP was targeted to the cytoplasm of the parasite, whereas T–GFP was targeted mainly to the PV, with some fluorescence also detected in the tubulovesicular network and the food vacuole. These results demonstrated that transport to the apicoplast is a two-step process, in which the protein must first enter the secretory pathway (signal peptide) and then be directed to the organelle (transit peptide). Western blot analysis of the apicoplast-targeted proteins also demonstrated that both S and T peptides are cleaved during transport.

In other organisms containing plastids such as algae and plants, the protein transport to this organelle first involves cleavage of the signal peptide, which happens during import into the ER. Subsequently, the transit peptide is then cleaved by a stromal-processing peptidase (SPP) at some point during translocation through the membrane of the plastid. In *P. falciparum*, the putative gene encoding the SPP has been identified (van Dooren *et al.*, 2002). In this study, a pulse–chase experiment was used to determine that the cleavage site of a the prosequence fused to GFP was 25 residues after the putative cleavage site of S peptide and just before the sequence LNRKN. It was also shown that the *P. falciparum* SPP catalytic region contains residues that are conserved in the SPPs of other organisms. After S peptide cleavage, the T peptide was completely degraded by a metalloprotease. In *P. falciparum*, this protease has been shown to be the metalloprotease falcilysin (Ponpuak *et al.*, 2007), which has also been shown to be involved in the hemoglobin digestion (Eggleson *et al.*, 1999). Through GFP tagging, it was demonstrated that falcilysin localizes to the apicoplast as well as the food vacuole, and that it can degrade the transit peptide only after it has been removed from the rest of the protein (Ponpuak *et al.*, 2007).

The presence of a signal peptide inside the prosequence of the apicoplast-targeted proteins strongly suggests they must pass through the secretory pathway on route to this organelle. Tonkin and coworkers demonstrated that the S peptide is not specific to apicoplast proteins (Tonkin *et al.*, 2006b). They replaced the S peptide of an ACP presequence–GFP fusion with the signal peptide of KAHRP and EBA-175, which are exported to the erythrocyte cytoplasm and the micronemes, respectively. GFP was still transported to the apicoplast, demonstrating the S peptide is canonical. They also showed that BFA does not alter this apicoplast targeting, demonstrating this apicoplast route to be extra-Golgi. These results also indicate a close association between apicoplast and ER and the possibility that they can be continuous. Similar results were obtained with *T. gondii* (DeRocher *et al.*, 2005). However, in this system the transit peptide cleavage is inhibited by BFA, whereas apicoplast-targeted transport remains insensitive to the drug.

4.2. Export to infected red blood cell cytoplasm and surface

The extensive modifications produced in the infected RBC by *P. falciparum* require a large number of parasite proteins to be placed in both the host cytosol and cell surface. As mentioned previously, the protrusions produced in the RBC membrane, termed knobs, are of high relevance in the establishment of pathology. Probably the most important element in this structure is the knob-associated histidine-rich protein (KAHRP). Important clues about the transport of KAHRP came from the study by Wickham and coworkers (2001). They demonstrated that a GFP fusion with the

N-terminal 60 residues is retained in the PV and that this process is BFA sensitive. These results suggested the first step of KAHRP transport occurs through the classical secretory pathway. However, translocation across the PV membrane was achieved only when GFP was fused with the N-terminal 123 residues, indicating other signals are involved. Interestingly, this longer GFP fusion accumulates in Maurer's clefts, suggesting this organelle is important for protein sorting and transport to the RBC membrane.

Maurer's clefts are irregularly shaped membranous compartments localized close to the RBC membrane, possibly interacting with the host cell cytoskeleton. It has been proposed that they originate from the PV (Spycher *et al.*, 2006), although they are probably not interconnected. Evidence suggests RBC surface antigen proteins such as PfEMP1 and RIFINs, and surface-associated proteins such as KAHRP and PfEMP3, transiently pass through Maurer's clefts before reaching their final destination (Khattab and Klinkert, 2006; Knuepfer *et al.*, 2005; Waterkeyn *et al.*, 2000; Wickham *et al.*, 2001).

The peptide sequences necessary for transport to RBC have been discovered. A bioinformatics approach allowed the identification a motif (R/KxLxE/Q), termed *Plasmodium* export element (PEXEL) or vacuolar transport signal (VTS) present in the N-terminal region of exported proteins (Marti *et al.*, 2004; Hiller *et al.*, 2004). GFP fusions with the protein N-terminal regions harboring this motif or mutated versions of it demonstrated its requirement for translocation across the PV membrane. The data from these studies were used to generate an algorithm, named ExportPred, which enabled the identification of novel families of exported proteins in *Plasmodium* species (Sargeant *et al.*, 2006).

The mechanisms of protein transport into the RBC cytosol are not fully characterized. It has been proposed that whereas soluble proteins such as KAHRP, HRP-2, and others diffuse across the RBC cytosol in the form of aggregates, integral membrane proteins such as PfEMP1 are transported in vesicles (Cooke *et al.*, 2004). The destinations of both protein classes seem to be Maurer's clefts (Haldar *et al.*, 2005). However, more recent data argue against this model, suggesting that PfEMP1 is transported as a protein complex (Knuepfer *et al.*, 2005). It has been demonstrated that membrane proteins such as PfEMP1 and Stevor require not only the S peptide and export motif, but also the transmembrane domain in order to be properly targeted to the RBC surface (Knuepfer *et al.*, 2005; Przyborski *et al.*, 2005). The first molecule involved in translocation of proteins to the RBC surface has just been characterized (Cooke *et al.*, 2006). The knockout of *Pfsbp1* disrupted translocation of PfEMP1 to the RBC membrane.

Despite these advances, many aspects of protein trafficking in malaria parasites are still obscure. Further studies may improve our understanding of both the signals and the cellular machinery involved in protein transport, which may also bring new targets for therapeutic intervention.

4.3. Cytoadherence

Severe malaria is a systemic disease, affecting the physiology of many organs, which may eventually lead to the death of the host. Some of its symptoms include lung edema, renal failure, severe anemia, hypoglycemia, metabolic acidosis, and cerebral malaria, progressing to coma (Kirchgatter and Del Portillo, 2005). The major cause of deaths in sub-Saharan Africa and in other regions seems to be cerebral malaria. This brain disorder is caused by the adhesion of RBCs infected with trophozoite and schizont stages of *P. falciparum* to cerebral endothelial cells, obstructing small vessels and, if also occurring in the visceral blood vessels, causing organ failure (Miller *et al.,* 2002). This phenotype, known as cytoadherence (Udeinya *et al.*, 1981), is absent in the other three species of *Plasmodium* that infect humans.

Cytoadherence depends on structural changes at the infected RBC surface caused by the parasite. The most important seems to be knob formation (Luse and Miller, 1971). It was first proposed that the main component of the knob structure is the knob-associated histidine-rich protein (KAHRP) (Kilejian and Jensen, 1977; Kilejian, 1979) and later, Crabb and colleagues functionally demonstrated that KAHRP is essential to knob development (Crabb *et al.*, 1997). The KAHRP gene was disrupted, which did not affect parasite progression in the erythrocytic cycle, but impaired knob formation and drastically reduced the cytoadherence under flow conditions. These data suggested that the role of the knob is probably to strengthen the interaction between the infected RBC and endothelial cells, allowing cytoadherence to occur at the flow conditions present in small vessels.

It is widely accepted that cytoadherence is a caused by the interaction of ligands of the RBC surface, most of them placed on the knobs, with endothelial receptors. The ligands can be parasite proteins such as PfEMP1, CLAG9, Stevor, RIFIN, or host erythrocyte-derived molecules such as modified band 3 (Craig and Scherf, 2001). Some of the endothelial receptors are cluster of cell determinant 36 (CD36), thrombospondin (TSP), intercellular adhesion molecule-1 (ICAM-1), and chondroitin-4-sulfate (CSA) (Baruch, 1999).

The parasite ligand considered most important for cytoadherence is PfEMP1 (Leech *et al.*, 1984). This protein is encoded by the *var* gene family, which is represented by about 60 members per haploid genome (Baruch *et al.*, 1995; Gardner *et al.*, 2002a; Su *et al.*, 1995). Each PfEMP1 member is composed of a variable number of Duffy binding-like domains (DBLs) and cysteine-rich interdomain regions (CIDRs), one transmembrane (TM) domain, and an acidic terminal segment (ATS). PfEMP1 is localized on the knob surface, exposing the DBLs and CIDR domains on the outside of the RBC, whereas the ATS domain stays on the cytoplasmic side. It has been demonstrated that these external domains are able to adhere to distinct

receptors (Craig and Scherf, 2001) and the switch of *var* gene expression determines a new adhesion phenotype (Smith *et al.*, 1995).

CLAG9 is another protein implicated in cytoadherence. *clag9* belongs to a family of subtelomeric genes with similar structure, harboring at least nine exons and a transmembrane domain (Holt *et al.*, 1999). The first evidence of its involvement with cytoadherence came from the observations that some parasite lines kept in culture for long periods lost their ability to cytoadhere to CD36 and that this was linked to a deletion on chromosome 9 (Bourke *et al.*, 1996; Chaiyaroj *et al.*, 1994; Day *et al.*, 1993; Shirley *et al.*, 1990). Functional demonstration was provided with both the knockout and the knockdown of *clag9*, using homologous integration and antisense RNA, respectively (Trenholme *et al.*, 2000; Gardiner *et al.*, 2000). In these studies, the knockdown and knockout lines had significantly reduced binding to CD36, indicating that CLAG9 is an important ligand to this endothelial receptor. However, more recently, CLAG9 has been redesignated as a merozoite rhoptry protein of the high molecular weight rhoptry (RhopH complex) family (Ling *et al.*, 2004). Thus, the significance of the cytoadherence data needs further clarification.

Some endothelial receptors have been characterized and their ligands determined (Craig and Scherf, 2001). It has been proposed that the adhesion process is composed of three sequential main stages: tethering, rolling, and stationary adhesion (Baruch, 1999; Ho and White, 1999). According to this model, although most receptors can support the first two stages, only CD36 and CSA can certainly support the last. Considering that CSA is the main receptor at the placenta (Fried and Duffy, 1996) and that CD36 is present on the endothelium, the latter is likely to be the most generally used receptor for cytoadherence to endothelial cells. The tissue specificity of adhesion can be attributed to other receptors. In fact, it has been demonstrated that CD36 binds to most parasite isolates (Hasler *et al.*, 1990), whereas other host cell receptors do not.

Some types of severe malaria are linked to adhesion to some specific receptors. Epidemiological studies demonstrated that isolates from patients with cerebral malaria preferentially adhere to ICAM-1 (Newbold *et al.*, 1999). The same study observed colocalization of ICAM-1 and infected RBCs in blood vessels of patients who had succumbed to cerebral malaria. On the other hand, CD36 binding could not be correlated with this type of severe malaria.

Although cytoadherence can be extremely harmful to the host, it provides the parasite a means to escape clearance by the spleen. However, by putting its ligands on the RBC surface, the parasite becomes a target for antibodies. To avoid this attack, ligand expression, especially of PfEMP1, is tightly regulated and this protein family switches between parasite generations, producing an antigenic variation phenotype. This model is accepted by many authors, as discussed by Borst and coworkers (1995). An alternative

model has been proposed (Saul, 1999): during human infection, *P. falciparum* may behave as a multicellular organism in which some cells (parasites) must die to allow the whole organism to survive. According to this model, the first function of the antigens expression on the surface of the RBC would be to induce an immune response, which would kill most of the parasites, decreasing their growth rates and allowing the host to survive long enough for the production of gametocytes and their transmission to the mosquito. Cytoadherence would be a fail-safe mechanism responsible for the spleen killing those parasites that did not express the ligands. Whatever model better reflects *P. falciparum* infection, cytoadherence is clearly an important target for therapeutic intervention, and a deeper knowledge of the elements involved in this process must be pursued.

5. Cellular Calcium Dynamics

Ca^{2+} signaling is known to modulate a wide range of functions in high and low eukaryotes (Berridge *et al.*, 2003). Whereas Ca^{2+} homeostatic mechanisms have been extensively studied in multicellular organisms, particularly in mammals, our understanding of Ca^{2+} handling and signaling in apicomplexans is relatively less complete. Indeed, these eukaryotic unicellular organisms are endowed with a few unusual Ca^{2+} homeostatic mechanisms that may even be explored as therapeutic targets (Nagamune and Sibley, 2006).

Here we briefly describe some of the most common methodologies employed for measuring Ca^{2+} in living cells, discuss a few basic concepts of the Ca^{2+} homeostatic mechanisms in eukaryotes, and then focus specifically on Ca^{2+} signaling mechanisms used by malaria parasites to survive and develop inside RBCs.

5.1. Fluorescent and chemiluminescent probes to study Ca^{2+} in living cells

The first reliable Ca^{2+} concentration ($[Ca^{2+}]$) measurement in intact living cells is probably that performed by Ridgway and Ashley (1967), and was obtained through the injection of aequorin, a Ca^{2+}-sensitive photoprotein, into the giant muscle fiber of barnacles. Aequorin was first isolated from the coelenterate *Aequorea victoria* (Shimomura *et al.*, 1962) and later its gene was cloned and expressed in different cell types. Aequorin can be microinjected (a time-consuming and complex procedure, best suited for giant cells) or, alternatively, can be expressed by transfection of the cDNA (only apoaequorin is produced, but the complete functional protein can be reconstituted

even in living cells simply by adding to the medium the prosthetic group coelentrazine) (Brini *et al.*, 1999; Brownlee, 2000).

A breakthrough in the field was the rational design by Tsien and colleagues of a family of fluorescent Ca^{2+} indicators and the invention of a methodology to trap them into cells without the need to microinject the cells (Grynkiewicz *et al.*, 1985; Minta *et al.*, 1989; Tsien, 1980; Tsien *et al.*, 1982). There are today many fluorescent Ca^{2+} probes available with different features and each of them has advantages and disadvantages that can be exploited for the specific needs of the experimenter. These dyes can be separated into different groups depending on the feature considered (i.e., ratiometric, in which Ca^{2+} binding to the dye causes a major change in the excitation or emission spectrum; and nonratiometric, in which Ca^{2+} binding causes only a change in fluorescence intensity), leading to blue, green, or red emission. The availability of dyes with different emission spectra is particularly important if two fluorescent probes are to be used in the same experiment (i.e., for measurement of Ca^{2+} and pH) (Takahashi *et al.*, 1999).

The different probes have distinct Ca^{2+} affinities, and this is reflected to their K_d (dissociation constant). Probes with low K_d values (high Ca^{2+} affinities) may offer brighter fluorescence, but are obviously saturated by relatively low $[Ca^{2+}]$. Such dyes are useful for monitoring Ca^{2+} in the cytoplasm, but are not useful for performing Ca^{2+} measurements within organelles, where either the ambient $[Ca^{2+}]$ is high under resting conditions (e.g., the ER) or can reach high levels during stimulation of the cells (e.g., the mitochondria) (Takahashi *et al.*, 1999). It should be noted that, with the notable exception of dyes targeted to the mitochondrial matrix, the trapping of these probes in the various cell organelles is variable and not specific.

The introduction of recombinant Ca^{2+} sensitive proteins was another major advancement in the measurement of Ca^{2+} dynamics in living cells. The major advantage of genetically encoded Ca^{2+} sensors is that targeting sequences can be introduced that are both necessary and sufficient to drive the expressed protein in any cellular organelle. The prototype recombinant Ca^{2+}-sensing protein aequorin cloning was achieved by Inouye *et al.* (1985) and aequorins targeted to different organelles or cytosolic regions are now available. Aequorin, although still used, and probably the probe of choice for some types of experiments, suffers from some disadvantages, such as the low level of emitted light, the irreversible oxidation of coelenterazine on photon emission, and so on. The next generation of Ca^{2+}-sensitive proteins is that designed by Tsien and coworkers, named Cameleons. The Cameleons are artificially designed fluorescent proteins, consisting of two GFP variants flanking the Ca^{2+}-binding protein calmodulin (CaM) and a CaM-binding domain (originally the M13 peptide derived from myosin light chain kinase), and take advantage of the phenomenon of fluorescence resonance energy transfer (FRET) (Pozzan *et al.*, 2003). Using the same approach initially

designed for aequorin, targeting sequences can be added to Cameleons to examine Ca^{2+} signals in specific subcellular regions or organelles.

5.2. Calcium-handling mechanisms in *Plasmodium*

As any other eukaryotic cell, malaria parasites maintain a low level of cytoplasmic Ca^{2+} (Garcia, 1999). This low level is essential for survival for two reasons: (1) if free Ca^{2+} in the cells increases to high levels for several minutes, the cell dies; and (2) at the same time, because the Ca^{2+} level outside the cell is 10,000-fold higher than inside, the cell uses this gradient to allow low amounts of Ca^{2+} into the cells for brief periods of time. These small and brief increases in Ca^{2+} inside the cells represent a general form of activation signal.

As discussed above, knowledge of the mechanisms involved in Ca^{2+} homeostasis has greatly increased, particularly in mammals and other higher eukaryotes. On the other hand, much less is known about this fundamental aspect of cell physiology in plants and low eukaryotes. Yet a vast number of cellular events both in plants (Goddard *et al.*, 2000) and lower eukaryotes are known to employ Ca^{2+}-based signaling pathways.

From the point of view of Ca^{2+} homeostasis, intracellular parasites were always treated as special cases, because they develop inside other cells and therefore it has been presumed that they are exposed to the nanomolar Ca^{2+} environment of the host cell cytoplasm. Here we focus on the mechanisms of Ca^{2+} handling by the protozoan parasite *Plasmodium*. In the following sections we briefly summarize a few specific features of these pathogenic microorganisms, emphasizing the more recent advances in the knowledge of Ca^{2+} signaling-dependent mechanisms required for them to invade, develop and survive inside host cells.

5.3. From genome to cell physiology

The sequencing of the *Plasmodium* genome (Gardner *et al.*, 2002a) and related studies from a number of laboratories, have identified in *Plasmodium* several genes encoding molecules that are presumably involved in Ca^{2+} signaling and handling.

The E-F hand (Ca^{2+}-binding motif) is present in several *Plasmodium* proteins (Aravind *et al.*, 2003; Rawlings and Kaslow, 1992). Regarding Ca^{2+} pumps and channels, the parasite possesses only one gene, PfATP6, encoding a sarcoplasmic reticulum ATPase (SERCA) (vertebrates possess three genes and several spliced variants; Nagamune and Sibley, 2006). PfATP6 of *Plasmodium* can be inhibited by artemisinins and thapsigargin (Eckstein-Ludwig *et al.*, 2003).

A Golgi-like Ca^{2+} ATPase (PfATP4) and a single Ca^{2+}/H^{+} exchanger were found in *Plasmodium*. Na^{+}/Ca^{2+} exchangers were not found in

apicomplexans (Nagamune and Sibley, 2006). Moreover, no inositol 1,4, 5-trisphosphate receptor (IP_3R) channel homologs have been found in *Plasmodium*, which indicates that a more primitive Ca^{2+} release channel may exist in these parasites (Nagamune and Sibley, 2006). Furthermore, P-type Ca^{2+} ATPases (Kimura *et al.*, 1993; Murakami *et al.*, 1990; Trottein and Cowman, 1995; Trottein *et al.*, 1995), the Ca^{2+}-binding or -dependent proteins such as CaM (Robson and Jennings, 1991), reticulocalbin (La Greca *et al.*, 1997), calcineurin (Dobson *et al.*, 1999; Kumar *et al.*, 2005), and several Ca^{2+}-dependent kinases (Zhao *et al.*, 1993, 1994a,b) have been reported to be present in the *Plasmodium* genome.

Interestingly, while a canonical protein kinase C has not been found in the genome, a receptor for an activated kinase C ortholog has been reported in *P. falciparum* (Madeira *et al.*, 2003). In addition to this information at the molecular level, physiological data indicate a major role for a Ca^{2+}-signaling pathways in many aspects of *Plasmodium* physiology. These include $^{45}Ca^{2+}$ flux measurements (Matsumoto *et al.*, 1987; McCallum-Deighton and Holder, 1992; Scheibel *et al.*, 1987; Wasserman and Chaparro, 1996) and inhibition by Ca^{2+} chelators and CaM inhibitors of the invasion–maturation process.

The requirement of extracellular Ca^{2+} for RBC invasion by *P. falciparum* was demonstrated by several laboratories (Johnson *et al.*, 1980; Matsumoto *et al.*, 1987; McCallum-Deighton and Holder, 1992; Tanabe, 1990; Wasserman, 1990). Moreover, the depletion of this extracellular ion prevents the completion of *P. falciparum* cycle in culture (Wasserman, 1990). Interestingly (McCallum-Deighton and Holder, 1992), it has been demonstrated that invasion of RBCs by *P. falciparum* is specifically dependent on Ca^{2+} because medium supplementation with other cations, such as magnesium, manganese, and zinc, does not restore the invasion capacity.

Pioneer work on *Plasmodium*-infected RBCs by Tanabe *et al.* (1982, 1989) and Krungkrai and Yuthavong (1983) demonstrated by $^{45}Ca^{2+}$ flux studies that the total Ca^{2+} content of RBCs increases after *Plasmodium* invasion. Ca^{2+} is also involved in the process of exflagellation (microgamete formation) in both *P. berghei* and *P. falciparum* (Kawamoto *et al.*, 1990, 1991).

5.4. Parasite organelles as intracellular Ca^{2+} pools

Extensive work in mammalian cells has provided conclusive evidence supporting the idea that the ER, mitochondria, and Golgi apparatus play essential, although different, roles in cellular Ca^{2+} homeostasis. In the ER (and its muscle counterpart the sarcoplasmic reticulum, SR), Ca^{2+} accumulation depends on the expression of specific Ca^{2+}-ATPases (SERCAs) that utilize the energy derived from ATP hydrolysis to pump Ca^{2+} from the cytoplasm into the lumen. The Golgi uses both SERCA and another Ca^{2+}

ATPase to accumulate Ca^{2+}, whereas mitochondria take up Ca^{2+} through a completely different mechanism, a cation channel, driven by the electrical potential, negative inside, across the inner mitochondrial membrane. Work has concentrated on some of the mechanisms of cross-talk between Ca^{2+} within organelles (the ER in particular) and the regulation of Ca^{2+} channels in the plasma membrane. It has been demonstrated that an integral membrane protein of the ER, STIM1, is capable of sensing the lumenal ER [Ca^{2+}] and, through a still largely mysterious mechanism, regulates the activity of a plasma membrane Ca^{2+} channel, named the Ca^{2+}-release-activated-Ca^{2+} channel (Lewis, 2007; Yeromin *et al.*, 2006; Zhang *et al.*, 2005).

The DoCampo laboratory was of the first to use fluorescent Ca^{2+} indicators to study Ca^{2+} homeostasis in unicellular parasites (they used *Trypanosoma cruzi* as a model) (DoCampo, 1993; DoCampo *et al.*, 1995; Lu *et al.*, 1998; Moreno and DoCampo, 2003).

Fluorescent dyes have been used to investigate Ca^{2+} homeostasis and signaling in *Plasmodium* (Adovelande *et al.*, 1993; Beraldo and Garcia, 2005; Beraldo *et al.*, 2005; Budu *et al.*, 2007; Garcia *et al.*, 1996, 1998; Gazarini and Garcia, 2003; Gazarini *et al.*, 2003; Gazarini and Garcia, 2004; Hotta *et al.*, 2000; Passos and Garcia, 1998; Varotti *et al.*, 2003). Convincing evidence for the existence of two distinct Ca^{2+} storage compartments in different *Plasmodium* species has been provided (Alleva and Kirk, 2001; Biagini *et al.*, 2003; Garcia, 1999; Garcia *et al.*, 1996, 1998; Passos and Garcia, 1998; Varotti *et al.*, 2003). One has characteristics similar to those of the ER in mammalian cells, and the other is rather typical of these parasites, that is, characterized by an acidic lumenal pH; both have sensitivity to IP_3 (Passos and Garcia, 1998). This latter acidic Ca^{2+} pool might be the food vacuole involved in hemoglobin digestion as well as in the antimalarial action of chloroquine. IP_3-mediated Ca^{2+} release from acidic, non-ER compartments has been reported also in mammalian cells (Gerasimenko *et al.*, 1996). Interestingly, Martin *et al.*, (1994) had reported phosphoinositide hydrolysis during exflagellation of the *Plasmodium* sexual stage gametocyte. In the same direction, Beraldo *et al.*, (2007) found that 2-APB blocks a IP_3 receptor, impairing melatonin-signaling effects in *P. falciparum*, thus reinforcing the concept that this second messenger plays a role in the parasite cell cycle.

As discussed above, a classical IP_3 receptor gene has not been identified in the genome of *Plasmodium*. However, on the basis of the functional evidence that IP_3 can release Ca^{2+} from *Plasmodium* Ca^{2+} stores and considering the phylogenetic distance between mammals and protozoans (and the fact that 60% of predicted proteins in the genome do not have similarity to any proteins in other organisms; Gardner *et al.*, 2002a), it is reasonable to assume that an IP_3 receptor is expressed in *Plasmodium*, but its sequence is probably quite different from that of higher eukaryotes.

As to the ability of these parasites to use other Ca^{2+}-mobilizing second messengers such as cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), it should be noted that cADPR and cADPR hydrolase and cyclase activities have been reported in lysates of *T. gondii*. These findings suggest that the second messenger cADPR, which evokes Ca^{2+} release from the ER by activating RyR receptors, may be part of the Ca^{2+} homeostatic mechanisms at least in *T. gondii*, but possibly also in other *Plasmodium* species (Chini *et al.*, 2005).

5.5. Surviving in low-[Ca^{2+}] parasitophorous vacuoles

A unique feature of intracellular parasites such as *Plasmodium* species, regarding Ca^{2+} homeostasis, is that the cytosol of a parasitized cell is low in Ca^{2+}. However, malaria parasites inside the RBC are not free, but are surrounded by a membrane, the PVM, which is derived from the invagination of the host cell plasma membrane and contains parasite lipids and proteins. Some proteins and perhaps lipids are secreted at invasion from the rhoptries (see above), but the majority are added *de novo* by the parasite as the PVM grows to accommodate the expanding parasite. The PVM of *P. falciparum* and *Toxoplasma gondii* have been reported as being molecular sieves with similar size exclusion limits, around 1.9 kDa (Desai and Rosenberg, 1997; Kirk, 2001; Desai *et al.*, 1993; Schwab *et al.*, 1994). The result is that small molecules can freely cross the PVM. These data suggest that the intravacuolar ionic composition is roughly equivalent to that of the host cell cytoplasm, that is, it should have a low [Ca^{2+}]. Such a situation is clearly in contrast to that encountered in most cells, which are exposed to an extracellular environment with millimolar [Ca^{2+}], and raises an important question: what strategies did the parasites develop to maintain Ca^{2+} within physiological limits? In particular, how do parasites keep their intracellular Ca^{2+} store compartments full and ready to signal?

We hypothesized that the mechanism invented by the malaria parasites to live within the RBC resides in the nature and structure of the PVM. The hypothesis was that this PVM encapsulates the parasite and creates around it a tiny, but essential, space (the PV) with a relatively high Ca^{2+} level. This ensures that the malaria parasite is constantly exposed to high Ca^{2+} levels and thus can use Ca^{2+} as a signal. To test this hypothesis we entrapped fluorescent Ca^{2+} dyes (MagFura-2 and Fluo-3) selectively in this space during RBC invasion by *Plasmodium* (Gazarini *et al.*, 2003). Confocal microscopy was then used to measure the intensity of the fluorescence signal, and was indeed able to demonstrate that the space encapsulated by the PVM contains a relatively high [Ca^{2+}].

The plasma membrane Ca^{2+} ATPase (PMCA) might be responsible for the maintenance of high [Ca^{2+}] in the PV. When *Plasmodium* invades an RBC, some of the erythrocyte Ca^{2+} pumps remain trapped at the PVM.

Rather than extruding Ca^{2+} out of the cell, the RBC PMCA pumps Ca^{2+} into the PVM around the parasites and maintains a $[Ca^{2+}]$ of ~40 μM in the intravacuolar space, creating a sufficiently high $[Ca^{2+}]$ environment in which *Plasmodium* parasites can preserve the Ca^{2+} content of their intracellular stores. It was also demonstrated experimentally that, if the Ca^{2+} level in the PV was reduced, the development of the parasites was impaired (Gazarini *et al.*, 2003).

Indirect evidence supports the idea that the $[Ca^{2+}]$ of the PV may vary during parasite development. For example, in measuring the ATPase activity by electron microscopy (by the formation of electrodense precipitates of lead phosphate) it was shown that at an early stage of development (ring forms, 16–20 h postinvasion) the Ca^{2+} ATPase activity was preserved in the erythrocyte membrane and at the PVM (Caldas and Wasserman, 2001). However, at the trophozoite stage (36–40 h postinvasion), the ATPase activity was preserved in the erythrocyte membrane, but was reduced at the PVM. In mature schizonts (45 h) the enzyme activity at the PVM was almost undetectable, although conserved at the erythrocyte membrane. The finding that the Ca^{2+} ATPase activity in the PVM diminishes with parasite maturation supports the idea that the parasite uses different strategies throughout its development to maintain an optimal ionic environment. Of interest, Martin *et al.*, (2005) reported more than 100 *Plasmodium* genes encoding membrane transport proteins.

5.6. Circadian rhythms, malarial infection, and Ca^{2+} signaling: modulation of *Plasmodium* cell cycle by tryptophan-related molecules

The lysis of RBCs by malaria parasites is a highly synchronized event in most *Plasmodium* species as particularly studied in *P. falciparum* and *P. chabaudi*. This mechanism facilitates the parasite evasion from the vertebrate immune system. Interestingly, *P. falciparum* in culture loses its synchrony (Trager and Jensen, 1976). Hawking and collaborators observed that the intraerythrocytic development of many malaria parasites is based on multiples of 24 h (Hawking, 1970; Hawking *et al.*, 1968, 1972). It has also been reported that host exposure to an inverted light/dark cycle also inverted the parasite intraerythrocytic development (i.e., schizogony occurred during the day rather than at night) (Arnold *et al.*, 1969; David *et al.*, 1978). This finding revealed the close link between the parasite life cycle and the host circadian rhythm and the resemblance of the former to a circadian rhythm. The connection between circadian rhythms of the host and the parasite cell cycle was reviewed by Garcia *et al.* (2001).

Many physiological processes of vertebrates are related to circadian rhythms and associated with the blood fluctuation of melatonin, a hormone produced in the pineal gland among other tissues. The gland responds to the

circadian fluctuation of melatonin in the bloodstream: during the dark phase secretion occurs and during the light phase plasma levels of melatonin are low. Melatonin is capable of synchronizing internal processes of the organism to the light/dark cycle (Pandi-Perumal *et al.*, 2006).

In addition to melatonin (Hotta *et al.*, 2000), the melatonin precursors *N*-acetylserotonin (NAS), tryptamine and serotonin also can induce an increase in cytosolic free Ca^{2+} in *P. falciparum* (Beraldo and Garcia, 2005) and *P. chabaudi* (Hotta *et al.*, 2003). Interestingly, *N*-acetyl-*n*-formyl-5-methoxykynuramine (AFMK), a product of melatonin degradation, also synchronizes *P. chabaudi* and *P. falciparum* parasites, evoking a [Ca^{2+}] increases in cytosolic calcium (Budu *et al.*, 2007). Using a phospholipase C blocker (U73122), Beraldo and coworkers (2005) showed that melatonin-mediated increases in cAMP could be impaired. Moreover, 3′,5′-cyclic monophosphate N^6-benzoyl/protein kinase A activator (6BZ-cAMP), a membrane-permeable analog of cAMP, was shown to increase cytosolic Ca^{2+} levels in the presence and absence of extracellular Ca^{2+}. Figure 3.2A–E shows confocal images of *P. falciparum* parasites loaded with Fluo-3 fluorescent dye for Ca^{2+} measurements. However, blocking PKA with peptide inhibitor of PKA (PKI) prevents rises in Ca^{2+} induced by 6BZ-cAMP (Beraldo *et al.*, 2005), thus showing that PKA is required for cAMP analog to elicit Ca^{2+} transients. An intracellular calcium channel is likely to be modulated by PKA and responsible for this calcium release. The data reported by Beraldo *et al.* (2005) show a complex cross-talk between Ca^{2+} and cAMP signaling resulting from melatonin cascade activation (Fig. 3.3).

Another host tryptophan-related molecule, xanthurenic acid, triggers gametogenesis in the mosquito-infective form (Billker *et al.*, 1998) and this effect appears to involve a rise in Ca^{2+} levels and activation of CDPK4 (Billker *et al.*, 2004). The upstream mechanism involved in transducing the signal of these molecules is awaiting characterization. The signal transduction guanine nucleotide protein (G protein) has not been identified in the *Plasmodium* genome. However, G protein-independent mechanisms of heptahelic receptor signaling have been reported (Brzostowski and Kimmel, 2001; Hall *et al.*, 1999). By using a bioinformatic approach, we have selected four genes from the *Plasmodium* genome database as candidates for a parasite serpentine receptor (L. Madeira, P. Galante, A. Budu, B. Malnic and C.R.S. Garcia, unpublished data). We are currently testing their ability to respond to melatonin and its derivatives.

5.7. Signal transduction-handling machinery in *Plasmodium*

Protein kinases are ubiquitous enzymes that are known to regulate a number of physiological processes. Many kinases are known to be directly or indirectly modulated by Ca^{2+}. Thus, knowledge of the basic mechanisms responsible for *Plasmodium* Ca^{2+} handling could provide new pharmacological

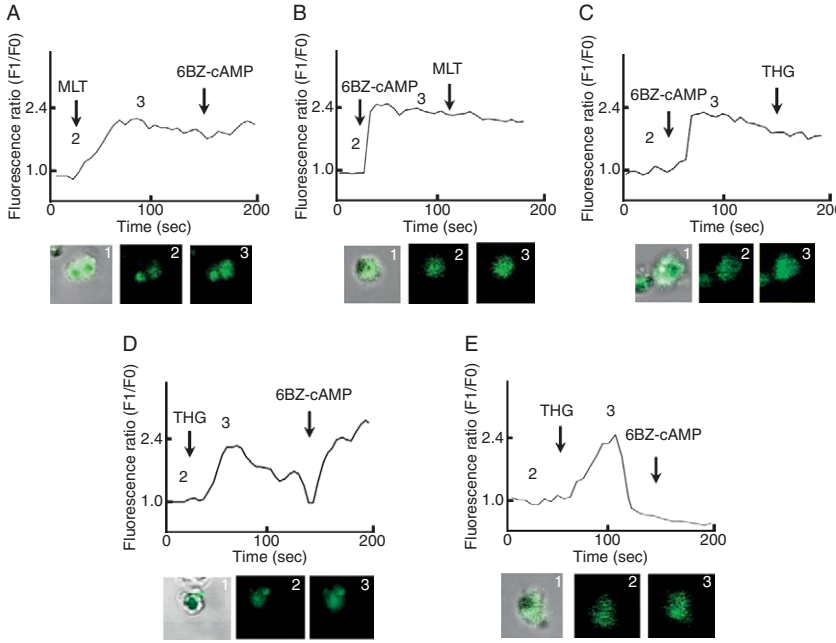

Figure 3.2 (A–E) Effects of 20 μ*M* 3′,5′-cyclic monophosphate N^6-benzoyl/PKA activator (6BZ-cAMP) and 100 n*M* melatonin on Ca^{2+} increase in the parasite cytoplasm. Addition of N^6-cAMP after melatonin does not evoke a further increase in calcium (A) as well as addition of melatonin after 6BZ-cAMP (B), suggesting a convergence of mechanisms for calcium release for these two molecules. Addition of 10 μ*M* thapsigargin (THG), a sarcoplasmic reticulum ATPase inhibitor, after 6BZ-cAMP in calcium-replete medium does not evoke a further increase in calcium, suggesting calcium release from the endoplasmic reticulum by 6BZ-cAMP (C). When the endoplasmic reticulum calcium pool is depleted with THG, a further increase in calcium evoked by 6BZ-cAMP is observed in Ca^{2+}-repleted medium (D), but no further increase is achieved by this same molecule in medium containing a calcium chelator, EGTA (E), suggesting a second mechanism of 6BZ-cAMP-induced calcium increase: calcium influx from the extracellular medium (Beraldo *et al.*, 2005). Reproduced from the *Journal of Cell Biology* 2005, 170:551–557 copyright © 2005, the Rockefeller University.

targets in malaria (Garcia, 1999; Kirk, 2001; Moreno and DoCampo, 2003). Here a few of the more recent findings on *Plasmodium*-expressed kinase activities are summarized, with particular emphasis on the potential modulation of the enzyme activities by Ca^{2+}.

Protein kinases of various *Plasmodium* species are rather divergent, at their primary sequence level, from kinases found in mammals. This divergence is probably due to the large phylogenetic distance between these two groups of organisms. This fact (which should reduce the potential effect on mammalian kinases of drugs affecting *Plasmodium* enzymes), along with the

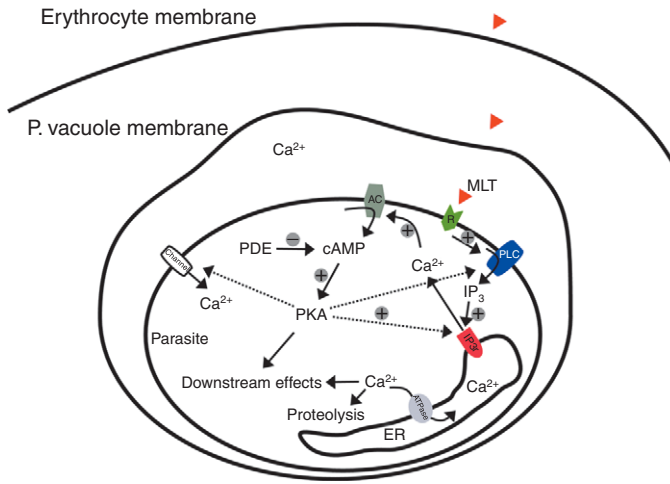

Figure 3.3 Model of signaling events in *Plasmodium*. AC, adenylyl cyclase; PLC, phospholipase C; PDE, phosphodiesterase; PKA, protein kinase A; ER, endoplasmic reticulum; R, hypothetical melatonin receptor (Beraldo *et al.*, 2005). Reproduced from the *Journal of Cell Biology* 2005, 170:551–557 copyright © 2005, the Rockefeller University.

crucial cellular roles that this class of proteins exert (Ward *et al.*, 2004), makes them a particularly interesting drug target (Doerig *et al.*, 2005). The completion of the *P. falciparum* genome (Gardner *et al.*, 2002a,b) contributed greatly to research regarding identification of *Plasmodium* protein kinases. A study by Ward and coworkers (2004) identified 85 genes encoding kinases. Yet another study, by Anamika and co-workers (2005), identified 118 genes corresponding to potential kinase sequences.

Although no receptor-linked tyrosine kinases have been found in the parasite genome, pharmacological evidence suggests that tyrosine kinase activity may be important during its intraerythrocytic development. Genistein and tyrphostin (both of which are considered to be selective inhibitors of protein tyrosine kinases) reduce parasitemia in *in vitro* experiments with *P. chabaudi*-infected erythrocytes (Dluzewski and Garcia, 1996; Gazarini and Garcia, 2003). A cGMP-dependent protein kinase was described in *P. falciparum*, displaying three domains for cGMP binding, contrasting with mammalian GMP kinases. *In vitro* phosphorylation of histone H1 and autophosphorylation levels increased when cGMP was added (Deng and Baker, 2002).

Regarding the calcium-dependent protein kinase (CDPK) family, it has been shown that *Plasmodium* expresses five proteins of this family, some of which (namely, CDPK2 and CDPK5) have unknown function (Kugelstadt *et al.*, 2007; Moskes *et al.*, 2004). It has been demonstrated that CDPK4 is activated during male gamete differentiation induced by xanthurenic acid,

a process that involves Ca^{2+} signaling (Billker *et al.*, 2004). CDPK3 is expressed during ookinete adhesion to the mosquito midgut (Ishino *et al.*, 2006). *Plasmodium* protein kinase B (PKB) is thought to have its activity regulated by Ca^{2+} (Vaid and Sharma, 2006). Indeed, Vaid and Sharma (2006) elegantly demonstrated that the enzyme activity of this kinase increased in a CaM dose-dependent manner. Moreover, this activity was dependent on Ca^{2+} given that EGTA, a Ca^{2+} chelator, inhibited the enzyme activity. Finally, CaM failed to activate PKB in a mutant lacking 21 residues in its N-terminal region, that is, that identified as a putative CaM-binding domain.

Mitogen-activated protein kinases (MAPKs) are known to participate in various processes within the cell, including regulation of cell cycle proteins, differentiation, and migration. Activation of these proteins results from a wide variety of stimuli, including hormones, cytokines, DNA damage, irradiation, and heat shock, among others (Garrington and Johnson, 1999). All MAPK pathways share a common three-component signaling cascade that consists of an MAPKKK (also known as MEKK or MKKK), an MAPKK (MKK or MEK), and an MAPK. Two MAPK homologs, Pfmap-1 and Pfmap-2, have been identified (Doerig *et al.*, 1996; Dorin *et al.*, 1999). MEK homologs have not been identified in the *Plasmodium* genome database. However, PfPK7, a protein expressed in asexual stages, sporozoites, and infected hepatocytes is peculiar in nature, as its C-terminal domain has homology with mammalian MAPKKs, but its N-terminal region has homology to fungal PKA lacking a typical activation site (Dorin *et al.*, 2005). Although this enzyme is able to phosphorylate a variety of substrates, data suggest that the enzyme is not an MAPKK ortholog. Altogether, there is strong evidence that the canonical three-component MAPK cascade is absent in *P. falciparum* (Dorin *et al.*, 2005).

In conclusion, dissecting the signaling pathways (Ca^{2+} targets and kinases) in malaria parasites is fundamental for the design of new drugs to break the pathogen cycle and fight the disease.

6. Molecular Biological Approaches to *Plasmodium* Studies

6.1. Transfection technology

More than half of the predicted genes in the sequenced *Plasmodium* genomes cannot be assigned to functions on the basis of sequence homology alone (Carlton *et al.*, 2002; Gardner *et al.*, 2002a; Hall *et al.*, 2005; and see http://www.sanger.ac.uk/Projects/P_*falciparum*/genome.overview.shtml).

Furthermore, the mechanisms controlling gene expression are still largely obscure. Bioinformatics and reverse genetics are two approaches

that have been used to elucidate gene function and regulation in a variety of sequenced organisms.

The transfection techniques are used to introduce exogenous DNAs or RNAs into eukaryotic cells. These molecules are designed to be able to recruit the cellular machineries of transcription, translation, replication, and eventual RNA degradation. Transfection can be subdivided into two categories: transient and stable. Transient transfections are used mainly to study the regions of DNA involved in transcription and/or translation regulation such as 5′ and 3′ untranslated regions (UTRs). The vector for the transient transfection is usually a plasmid with a reporter gene, under the control of the regulatory regions of interest. In the stable transfection, a permanently transformed cell line is cloned, which eliminates much of the variation inherent to a transient transfection assay. A vector used for stable transfection must have a marker, which generally encodes a gene that confers resistance to a drug that can be used for positive selection of transformed cells. Other elements, such as target integration sequences and additional expression cassettes, might also be present. This technique allows for the creation and complementation of mutants, as well as for the overexpression of proteins. Thus, the stable transfection is far more often used to study gene function, where the phenotype of the transformed cell lines can be analyzed and the function of the genes inferred.

6.2. Transfection in *Plasmodium* species

In malaria parasites, transient transfection was first reported in 1993 by electroporating gamete and zygote stages of *P. gallinaceum* with a plasmid harboring a luciferase (*luc*) reporter gene fused with the *Plasmodium pgs28* gene and its 5′ and 3′ flanking regions (Goonewardene *et al.*, 1993). Transient transfection of *P. falciparum* specifically was established in 1995 by electroporating ring-stage parasites with plasmids harboring the chloramphenicol acetyltransferase (*cat*) reporter gene under the control of the 5′ flanking regions of histidine-rich protein 3 (*hrp3*) or heat shock protein 86 (*hsp86*) genes and the 3′ UTRs of *hrp2* or *hsp86* (Wu *et al.*, 1995). This study provided the first characterization of promoters active in the intraerythrocytic stages, which allowed for the development of additional stable transfection vectors later on. In the same year, stable transfection of *P. berghei* was obtained, using the dihydrofolate reductase thymidylate synthase gene (*dhfr-ts*) mutated to confer resistance to drug pyrimethamine. Stable transfection of *P. falciparum* was achieved with either the *Toxoplasma gondii* or *P. falciparum dhfr-ts* gene mutated to confer resistance to pyrimethamine (Crabb and Cowman, 1996; Wu *et al.*, 1996).

In these studies, integration of the plasmids into the parasite genome by homologous recombination was observed. Whereas in *P. falciparum* this happened through a single crossover event, in *P. berghei* the plasmids

could be integrated by both single and double crossovers. Homologous integration of DNA opened the door to the possibility of generating gene knockout parasite lines either by integrating a selectable marker cassette directly into a gene, thus disrupting its expression (single crossover), or by completely replacing a targeted gene with cassette containing a selectable marker (double crossover). Nonhomologous recombination has also been observed (Crabb and Cowman, 1996; Miller *et al.*, 2002), although it has not been reported again and is probably a rare event.

The transfection methodology for other species of the genus *Plasmodium* has already been developed such as for *P. knowlesi* (van der Wel *et al.*, 1997), *P. yoelii* (Mota *et al.*, 2001), *P. cynomolgi* (Kocken *et al.*, 1999), and *P. vivax* (Pfahler *et al.*, 2006). Some of the plasmids successfully used in these studies contained promoters and terminators heterologous to the species transfected, demonstrating an absence of species barrier and the conservation of regulatory regions in the genomes of different parasites of the same genus.

More recently, some new selectable markers have been developed. The human dihydrofolate reductase gene (*hdhfr*) confers resistance to pyrimethamine and also to the drug WR99210, thus allowing transfection of parasite strains that have become resistant to pyrimethamine. This double-selectable marker also allows for the complementation of parasites already transfected with the first marker *dhfr* (Fidock and Wellems, 1997). The blasticidin S deaminase (BSD) (Mamoun *et al.*, 1999), neomycin phosphotransferase II (NEO) (Mamoun *et al.*, 1999), and puromycin-*N*-acetyltransferase (PAC) (de Koning-Ward *et al.*, 2001) genes have also been developed as selectable markers as they confer resistance to the drugs blasticidin S, geneticin (G418), and puromycin, respectively. *hdhfr* and *bsd* continue to be the most widely used in *P. falciparum*, whereas *dhfr* from *T. gondii* is most widely used in *P. berghei*.

6.3. Knockout

Single-crossover integration has allowed the knockout and allelic replacement of many genes, shedding light on many aspects of the biology of the parasites. These include genes involved in drug resistance, hemoglobin processing, invasion, translation, sexual differentiation, gamete development, protein trafficking, and others (Table 3.1). However, there are at least two important drawbacks to knockout by single-crossover integration. First, after integration, the plasmid backbone is retained in the disrupted gene locus, making it challenging to later knock out other genes in the same parasite line, using a different selectable marker. This is because the first integrated plasmid backbone oftentimes provides a larger target for homologous recombination of the second plasmid rather than the intended second gene targeted for knockout. Second, if the knockout causes a deleterious phenotype, the parasites with the plasmid in its episomal form will always

grow faster, which may impair the selection of parasites with the genomically integrated plasmid. To overcome these difficulties, a more complex strategy for double-crossover integration, using a negative selection marker in *P. falciparum*, was developed (Duraisingh *et al.*, 2002). The thymidine kinase gene (*tk*) from herpes simplex virus was used as this negative selectable marker. The *tk* gene confers susceptibility to the nucleoside analog ganciclovir, by converting it to a toxic metabolite. The *tk* gene was used together with the positive selectable marker *hdhfr* to create a positive/negative selection system. Thus, parasites with the episomal plasmid were resistant to WR99210, but susceptible to ganciclovir. Only integration by a double crossover removes the negative cassette, together with the plasmid backbone and the target sequence in the genome, generally the gene to be knocked out, leaving the positive cassette in the genome locus and rendering parasites insensitive to ganciclovir but still resistant to WR99210. This strategy has now become the main approach to knockout genes in *P. falciparum*, allowing the generation of mutant parasite lines with deleterious phenotypes (Table 3.1). This method has been further improved with a chimeric gene consisting of the *Saccharomyces cerevisiae* cytosine deaminase (ScCD) and uracil phosphoribosyltransferase (ScUPRT) genes fused. This new negative-selectable marker proved to be much more potent than *tk*, facilitating even more the generation of knockouts by double-crossover homologous recombination (Maier *et al.*, 2006).

Although the negative/positive selection strategy permits the knockouts that cause deleterious phenotypes, it cannot be applied to essential genes. The only way to knock out essential genes in a haploid organism such as *Plasmodium* is, during its blood stages, to do so with an inducible system. The basis for such a system in *P. falciparum* has been established (Meissner *et al.*, 2005). It was demonstrated that *T. gondii* synthetic *trans*-activators are recognized by *P. falciparum* transcription machinery and that regulated reporter gene expression can be achieved in parasite lines expressing these *trans*-activators when they are transfected with plasmids harboring reporter genes under the control of minimal promoters possessing a tetracycline operator. Improvements in this system might allow the conditional knockout of essential genes and the expression of toxic products that cause a dominant-negative phenotype.

6.4. Analysis of Transcription in *Plasmodium*

6.4.1. Transcriptional regulation and genome-wide mRNA studies

Transcriptional regulation of gene expression consists of complex interactions between basal transcriptional machinery, transcription factors, *cis*-regulatory promoter elements, and higher order chromatin structure. A major challenge for molecular biology in the coming decades is to identify these various transcriptional components and decipher how their interactions

contribute to the manifestation of phenotypic traits. This is especially pertinent to disease organisms such as *P. falciparum*, where understanding precisely how transcription is controlled may lead to the identification of cellular targets for the rational development of new drug and vaccine therapies. However, despite many years of research and the continued heavy burden of malaria on global human health, our understanding of the transcriptional mechanisms regulating gene expression in malaria parasites is far from comprehensive.

To date, information regarding the nature of transcriptional regulation mechanisms in *P. falciparum* has come from three general lines of research. These include (1) gene-by-gene studies of *P. falciparum* promoters, using reporter genes and electrophoretic mobility shift assays (EMSAs); (2) genome-wide measurements of steady state mRNA levels for both the vertebrate and insect stages of the complex parasite life cycle; and (3) *in silico* prediction of both *trans*- and *cis*-acting components of the transcriptional machinery from analysis of the *P. falciparum* genome sequence and accompanying genome-wide data sets. Herein, we review the major findings these avenues of research have yielded with particular emphasis placed on the importance that integrative analyses of genomic sequence and genome-wide data sets will continue to play in deciphering the nature of transcriptional regulation in malarial parasites.

6.4.2. Gene-by-gene studies

The most detailed knowledge of *P. falciparum* transcriptional control mechanisms has come from gene-by-gene studies using EMSAs and both transiently transfected and stably transformed reporter gene parasite lines. Despite a general lack of promoter sequence conservation between *P. falciparum* and other sequenced eukaryotes, studies using these approaches have shown that transcription in *P. falciparum* appears to be predominantly monocistronic and driven by classical eukaryotic bipartite-structured promoters (Alano *et al.*, 1996; Horrocks *et al.*, 1996; Mbacham *et al.*, 2001; Tosh *et al.*, 1999). In one early example, a 5-base pair sequence was found to be critical for proper intraerythrocytic stage transcription of glycophorin-binding protein 130 (*gpb130*), using promoter deletion mapping and EMSAs (Horrocks and Lanzer, 1999). In a similar contemporary study, the upstream promoter regions important for proper transcription of the early sexual development genes *pfs16* and *pfs25* were identified by deletion mapping, using transient transfection of chloramphenicol acetyltransferase (CAT) and luciferase reporter genes (Dechering *et al.*, 1999). By EMSA, these authors also demonstrated specifically that the sequence element AAGGAATA upstream of *pfs16* is bound in a sexual stage-specific manner by an undetermined protein called PAF-1 (Dechering *et al.*, 1999). Other, more recent studies using the same general approaches have yielded similar results for other loci including the identification of a 24-base pair sequence

important for the regulation of the phospholipid metabolism gene CDP-diacylglycerol (Osta *et al.*, 2002), the demonstration that the sequence element CAGACAGC is important for sexual stage promoter activity of *pgs28* in the related avian malaria parasite *P. gallinaceum* (Chow and Wirth, 2003), and the elucidation of various promoter elements key for the complex transcriptional regulation and gene-silencing mechanisms of *P. falciparum* multicopy gene families, specifically the *var* gene family.

6.4.3. Epigenetic control of gene expression

A number of organisms possess gene families whose expression is strictly controlled by different and, in many cases, largely unknown mechanisms. Examples of variant gene families include those encoding virulence factors involved in immune evasion [such as *Giardia lamblia* (Nash, 2002) and *Trypanosoma brucei* (Pays, 2005)], odorant receptors (Serizawa *et al.*, 2000), and immunoglobulins (Bergman and Cedar, 2004). In the late 1990s, two independent groups found that members of the *P. falciparum* variant antigen-encoding family of the *var* genes (Su *et al.*, 1995) are controlled by an allelic exclusion mechanism (Chen *et al.*, 1998; Scherf *et al.*, 1998). It is believed that *var* transcription starts at virtually all 50–60 *var* loci contained in the haploid genome, and that successful production of polyadenylated transcripts is then rapidly limited to one promoter (Scherf *et al.*, 1998; Kyes *et al.*, 2007). The *var* transcript of this active site encodes the variant antigen responsible for the adhesive phenotype of the infected red blood cell until the middle of the trophozoite stage and after that, perhaps coinciding with the onset of replication, this promoter is silenced. The epigenetic context appears to play a pivotal role in the recruiting of active promoters to the *var* transcription-competent site. Whereas active subtelomeric promoters associate with acetylated histone 4 but not PfSIR2 (a histone deacetylase), their silenced counterparts do (Freitas-Junior *et al.*, 2005) and the methylation of Lys-9 in plasmodial histone 3 (Chookajorn *et al.*, 2007) is a landmark for silenced *var* promoters. As for many other organisms a detailed histone code that defines active and inactive chromatin has yet to be elucidated. The *var* gene transcription pattern does not change in subsequent reinvasions, unlike other polymorphic genes (Preiser *et al.*, 1999) involved in erythrocyte infection, and the dominant transcript is usually maintained over many generations (Horrocks *et al.*, 2004; Roberts *et al.*, 1992). In higher eukaryotes, DNA methylation at promoter regions is a common feature and the feedback of methylated DNA and methylated histones may explain how these organisms maintain the epigenetic status of chromatin after replication. However, no DNA methylation occurs in *P. falciparum* (Scherf *et al.*, 1998), even at a low level (Choi *et al.*, 2006), and all the information for silencing or activation of loci must depend on factors associated with these loci, perhaps only dependent on histone modification. As for other organisms, it is currently unknown how this is accomplished in molecular terms.

Reminiscent of the control of variant surface gene (*vsg*) expression in *Trypanosoma brucei*, *var* gene transcription in *P. falciparum* is believed to occur at a defined site in the nuclear periphery that is free from condensed chromatin (Ralph *et al.*, 2005b). Whereas in *T. brucei vsg* expression is mediated by RNA polymerase I (Pol I) present in the nucleolus (Navarro and Gull, 2001), *var* genes are transcribed by RNA Pol II (Kyes *et al.*, 2007). Deitsch and collaborators analyzed factors that influence the transcription or silencing of *var* promoters. These authors found that the *var* intron, a relatively conserved sequence between the otherwise highly variant open reading frames (ORFs) of *var* genes, was responsible for silencing of adjacent *var* promoters (Deitsch *et al.*, 2001; Frank *et al.*, 2006). Also, an intrinsic bidirectional promoter function was described for this element (Gannoun-Zaki *et al.*, 2005). It can then be hypothesized that transcription from the intron leads to the accumulation of double-stranded RNA (dsRNA) transcripts that may, in analogy to the centromere of *Schizosaccharomyces pombe* (Volpe *et al.*, 2002), lead to the silencing of loci. However, a microarray-based analysis of sense and antisense transcripts from *var* loci did not reveal any consistent pattern for either silent or active *var* loci (Ralph *et al.*, 2005a). In addition, there are examples of *var* genes with truncated introns that lost the promoter-critical sequence motifs and the corresponding *var* genes are not transcribed. By constructing a complete *var* locus driving drug resistance expression, Voss and colleagues showed that the intron is dispensable for silencing and that the *var* promoter itself contained all the information necessary for activation and silencing (Voss *et al.*, 2006). Perhaps, the function of the *var* intron lies in the rapid and efficient silencing of loci (Deitsch *et al.*, 2001; and U. Gölnitz and G. Wunderlich, unpublished observations), possibly by recruiting unknown silencing factors. *var* promoters are somewhat promiscuous regarding their participation in the allelic exclusion mechanism: in stable (episomal) transfectants, *var* promoters can drive the constitutive expression of reporter genes independent of the remaining genomic *var* promoters (Frank *et al.*, 2006; and U. Gölnitz and G. Wunderlich, unpublished observations). Another example is the *var1csa* locus, which encodes a PfEMP1 with CSA adhesive domains (Buffet *et al.*, 1999). This gene is highly conserved in many strains (Winter *et al.*, 2003) and also transcribed (Kyes *et al.*, 2003), but is not translated in many cases, indicating that posttranscriptional events are important for the effective translation of *var* transcripts.

6.4.4. Transcriptome studies

The availability of *P. falciparum* transcriptome data sets has created new opportunities for understanding parasite transcriptional regulation that extends beyond the one-gene-at-a-time perspective. Even before the publication of the *P. falciparum* genome sequence in 2002, several semiglobal mRNA studies were conducted, using a variety of methods including serial

analysis of gene expression (SAGE) (Munasinghe *et al.*, 2001; Patankar *et al.*, 2001), subtractive and subtractive suppressive hybridization (Dessens *et al.*, 2000; Fidock *et al.*, 2002), cDNA libraries (Watanabe *et al.*, 2002, 2004), and genomic library- and cDNA-derived spotted microarrays (Ben Mamoun *et al.*, 2001; Hayward *et al.*, 2000). More recently, transcriptome analyses of the intraerythrocytic, gametocyte, and sporozoite stages, using both short- and long-oligonucleotide microarray platforms designed on the basis of genome sequence information, have provided even higher resolution information regarding *P. falciparum* steady state transcript levels (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003; Llinás *et al.*, 2006; Young *et al.*, 2005). Collectively, these studies have shown that *P. falciparum* mRNA levels tend to follow a highly ordered, relatively simple cascade of gene expression throughout both the intraerythrocytic and gametocyte developmental stages. Using a 70-mer spotted oligonucleotide array, an analysis of the 48-h intraerythrocytic cycle at 1-h resolution showed that the majority of genes are expressed during these stages and that more than 75% of these genes are activated only once (Bozdech *et al.*, 2003). In a similar study using a 25-mer high-density oligonucleotide microarray, transcripts were detected in the intraerythrocytic, sporozoite and gametocyte stages for ~88% of predicted *P. falciparum* genes (Le Roch *et al.*, 2003). These gene expression patterns have also been shown to conserved across geographically distinct strains of *P. falciparum* as demonstrated by profiling of intraerythrocytic stages of 3D7 (Netherlands), HB3 (Honduras), and Dd2 (Indochina) parasites (Llinás *et al.*, 2006).

The quantitative data on transcript levels obtained from these genome-wide mRNA profiling studies have also made possible in-depth statistical analyses of transcript coexpression for prediction of gene function. For example, transcripts encoding proteins possessing common functions or part of common multiprotein complexes have been shown to be coexpressed with significantly greater frequency than could be expected by chance (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003; Llinás *et al.*, 2006; Young *et al.*, 2005). This property has also been observed in other eukaryotic organisms such as *S. cerevisiae*, where it has been used to predict gene function by the principle of guilt by association (GBA) (Walker *et al.*, 1999). GBA states that if one can identify uncharacterized genes that possess expression patterns similar to genes for which function is known, one can rapidly make predictions that these uncharacterized genes may also be involved in similar biological processes as the characterized genes. This approach proved particularly useful for transcriptome-based prediction of gene function in *P. falciparum*, where approximately 60% of the genes have no known function based on experimental evidence or sequence homology (Gardner *et al.*, 2002a). By combining GBA and a *k*-means clustering approach to the *P. falciparum* life cycle microarray data, 15 clusters enriched for genes involved in various processes such as DNA replication, cell invasion,

and sporozoite development were identified (Le Roch *et al.*, 2003). This strategy was subsequently refined, using a more robust semisupervised algorithm called ontology-based pattern identification, which used gene ontology (GO) annotations (Ashburner *et al.*, 2000) to guide the generation of 380 higher resolution clusters for more precise gene function prediction (Young *et al.*, 2005; Y. Zhou *et al.*, 2005).

Beyond using gene coexpression data to predict gene function, another aim of genome-wide transcript profiling in *P. falciparum* has been to identify the cellular targets of antimalarial drugs through transcriptional response profiling. This strategy has been successfully employed to identify genes encoding components of pathways targeted by various small molecules in *S. cerevisiae* (Bro *et al.*, 2003; Cinato *et al.*, 2002; Zakrzewska *et al.*, 2005). Early SAGE studies analyzing *P. falciparum* transcriptional responses to chloroquine suggested that such an approach might also be used to determine the cellular targets of various antimalarials in *Plasmodium* species (Gunasekera *et al.*, 2003). However, subsequent efforts have repeatedly demonstrated that although *in vitro*-cultured parasite populations exposed to various drug and environmental stressors exhibit reproducible transcriptional responses, these changes tend to be weaker and less pathway specific than those observed in *S. cerevisiae* (M. Llinás, personal communication; E. Winzeler, unpublished results). For example, transcripts in parasites exposed to heat shock for 1 to 2 h as measured by Northern blotting (Militello *et al.*, 2004) and microarray hybridization seldom exceed greater than a 2-fold change in levels. Although others have observed by microarray hybridization potentially stronger *P. falciparum* transcriptional responses to heat shock, these changes also appear not to reflect much specificity beyond a general apoptosis-like parasite response to stress (Oakley *et al.*, 2007). Thus, malaria parasites, which reside in relatively buffered environments of vertebrate and insect hosts, may not possess the transcriptional feedback mechanisms other free-living microorganisms rely on in order to effectively respond in a specific manner to a diverse array of environmental changes in pH, temperature, and osmolarity. It remains to be seen whether additional genome-wide profiling of transcript or protein responses to drug pressure will prove successful in identifying the cellular targets of antimalarials in *P. falciparum*.

6.4.5. Bioinformatic approaches

The completion of the *P. falciparum* genome promised to accelerate the rate of progress of malaria research, which historically had been encumbered by a lack of robust genetic tools. However, despite shedding light on genes important for various processes such as metabolism and immune evasion, initial analyses of the *P. falciparum* genome sequence disappointingly provided few insights into the nature of the malaria parasite transcriptional machinery. First pass BLAST searches (Gardner *et al.*, 2002a) and subsequent

profile-hidden Markov model (HMM) analyses of the exceptionally AT-rich *P. falciparum* genome (~80.4%) identified far fewer transcription-associated proteins than had been found in other sequenced eukaryotes of similar genome size (Coulson *et al.*, 2004). Likewise, queries for conserved *cis*-regulatory DNA elements identified in other eukaryotic systems and annotated in databases such as TRANSFAC (Matys *et al.*, 2003) revealed that *cis*-regulatory elements seem to be largely diverged in *P. falciparum* (Kumar *et al.*, 2004) (E. Winzeler, unpublished results).

Since these first studies, more sophisticated analyses of the *P. falciparum* genome sequence have proven more successful in identifying both putative *trans*- and *cis*-acting elements of the parasite transcriptional machinery. Regarding *trans*-acting factors, two-dimensional hydrophobic cluster analysis (HCA) combined with profile-based search methods (PSI-BLAST) identified more general transcription factors, suggesting transcriptional regulatory mechanisms may be more prevalent in *P. falciparum* than early genome analyses suggested (Callebaut *et al.*, 2005). In this study, the transcription-associated factors TFIIA (large and small subunits), TFIIE (β-subunit), TFIIF (β-subunit), TFIIH (p62 and TFB1), and TFIID (TAF1, TAF2, TAF7, and TAF10) were predicted. Aside from TBP, which has been cloned and characterized (Ruvalcaba-Salazar *et al.*, 2005), none of these predicted factors have been experimentally investigated to date.

More progress has been made in *cis*-regulatory element discovery through the application of various algorithms such as MEME (Bailey and Elkan, 1994), AlignACE (Hughes *et al.*, 2000), MDScan (Liu *et al.*, 2002), and Weeder (Pavesi *et al.*, 2001, 2004). These background modeling-based *in silico* approaches identify putative *cis*-regulatory elements as sequence motifs that occur in the promoter regions of coexpressed or functionally related genes in greater frequency than would be expected if a random set of promoter regions were considered (i.e., the background). Millitello and coworkers used the algorithm AlignACE to analyze the promoter regions upstream of a hand-selected set of heat shock genes to successfully identify an enriched element they termed the G-box (Militello *et al.*, 2004). Deletion mapping of the *hsp86* promoter subsequently demonstrated the biological relevance for this element, as loss of the G-box element significantly reduced reporter gene activity (Militello *et al.*, 2004). This G-box motif was also found to be conserved upstream of *hsp86* homologs in the *Plasmodium* species *P. y. yoelii*, *P. berghei*, and *P. vivax*, thus adding even further support to its functional role (Militello *et al.*, 2004). In a broader study using a similar approach, van Noort and coworkers identified 28 putative regulatory elements by applying AlignACE to gene clusters generated from microarray expression data and refined with a phylogenetic footprinting information (van Noort and Huynen, 2006). These authors also observed that in many instances four or five different regulatory elements were found in the promoter of a single gene, suggesting that

although there may be a reduced number of transcription factors present in *P. falciparum*, complex transcriptional outputs may yet still be achieved through combinatorial regulation by these *trans*-acting factors (van Noort and Huynen, 2006).

Collectively, the results of these bioinformatic studies demonstrate that even when applied to nucleotide-biased genome such as that of *P. falciparum*, *in silico* methods hold much promise regarding the elucidation of transcriptional machinery components. Further customization of these methods for specific analysis of the *P. falciparum* genome, such as incorporating the use of empirical sequences rather than statistical background modeling and utilizing transcription start site information from apicomplexan cDNA databases such as Comparasite (Watanabe *et al.*, 2007), will serve to further improve the overall accuracy of *in silico cis*-regulatory element prediction in *P. falciparum*.

6.4.6. Future of malaria transcription research

The next major challenge for transcriptional research in malaria is to move beyond just biologically validating putative *cis*-regulatory elements by reporter gene assays and EMSAs, to actually identifying the *trans*-acting factors that bind these regulatory elements. To date, there have been few instances in malaria research of the identification of *trans*-acting factors that bind specific *cis*-regulatory sequences (Boschet *et al.*, 2004; Ruvalcaba-Salazar *et al.*, 2005; Voss *et al.*, 2002). In the future, DNA affinity purification of the *trans*-acting factors from parasite nuclear extracts followed by identification using mass spectrometry will play a major role in identifying other DNA-binding proteins with affinities for specific sequence elements.

With the advent of higher density *P. falciparum* microarrays, chromatin immunoprecipitation followed by microarray hybridization (chIP/chip) will also become important for empirically validating protein–DNA interactions. In *S. cerevisiae*, chIP/chip has been used to successfully profile binding sites for a number of transcription factors (Iyer *et al.*, 2001; Ren *et al.*, 2000). The application of chIP/chip to *P. falciparum* will be less straightforward as it is not clear which *P. falciparum* proteins predicted to contain putative DNA-binding domains are best suited for inclusion in a chIP/chip assay. As a result, priority should be placed on those proteins identified as likely to be involved in chromatin-associated processes from parallel data sets such as high-throughput protein–protein interaction network studies (LaCount *et al.*, 2005). Furthermore, chIP/chip also has the potential to greatly accelerate the characterization of higher order chromatin structure such as modified histones as suggested by some studies to be important for the epigenetic regulation of transcriptional control in *P. falciparum* (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005).

7. Concluding Remarks

Malaria research has yielded significant advances. Sequencing of the *Plasmodium* genome as well as the development of transfection methodology has enhanced our ability to decipher malarial protein function. However, there are still areas of malaria research for which further technological development is needed in order for us to best test hypotheses generated from the vast amount of sequence data now available. For example, a better system for the protein expression of AT-rich *Plasmodium* proteins would be extremely beneficial for advancing many areas of malaria research. Tools that enable us to better understand how the parasite decodes environmental information through the use of second messengers such as Ca^{2+} and cAMP also need to be refined. Furthermore, as more information is gleaned from genome-wide studies of protein and mRNA dynamics, it is becoming more and more clear that although traditional promoter-based transcriptional regulation exists in *P. falciparum*, it is only one component of a larger repertoire of gene expression-regulatory mechanisms employed by the parasite. Comparisons of transcriptome and proteome have shown that for many gene families a significant delay exists between peaks in mRNA and protein levels, suggesting that posttranscriptional mechanisms such as translational repression may be utilized widely by the parasite to regulate gene expression (Le Roch *et al.*, 2004). Similarly, mRNA decay rates appear to vary across the *P. falciparum* intraerythrocytic cycle, adding yet another level of complexity to transcriptional regulation (J. Shock *et al.*, 2007). It is insights such as these that will continue to usher malaria research into a new era of discovery, in which integration of genome sequence with genome-wide data sets will play as critical a role in advancing understanding of parasite transcriptional regulation as traditional experimental methods alone have done in the past.

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CHAPTER FOUR

Role of Nuclear Lamins in Nuclear Organization, Cellular Signaling, and Inherited Diseases

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Contents

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Abstract

Lamins are the major architectural proteins of the nucleus and are essential for nuclear integrity and assembly. Lamins are also involved in the organization of nuclear functions such as DNA replication, transcription, and repair. Mutations in the human lamin genes lead to highly debilitating genetic diseases that affect a number of different tissues such as muscle, adipose, and neuronal tissues, or cause premature aging syndromes. The observed interactions of lamins with inner nuclear membrane proteins, chromatin, and various regulatory factors have given important insights into the role of lamins in cellular processes and tissue-specific signaling pathways.

Key Words: Nuclear envelope, Lamin, Muscle differentiation, Adipocyte differentiation, Aging, DNA repair, Laminopathy.

1. Introduction

The nucleus in a eukaryotic cell is demarcated from the cytoplasm by the nuclear envelope. In most vertebrate and invertebrate species, the inner envelope consists of three prominent components: (1) the inner and outer nuclear membranes separated by a lumenal space; (2) the nuclear pore complexes (NPCs), which are macromolecular assemblies embedded in the nuclear envelope and mediate nucleocytoplasmic transport of macromolecules; and (3) the nuclear lamina, which forms a fibrous network beneath the inner nuclear membrane. The outer nuclear membrane is continuous with the rough endoplasmic reticulum, but the protein components of these two membranes are significantly different. A novel class of actin-binding proteins that has been identified in the outer nuclear membrane is the nesprin family of proteins (Starr and Han, 2003). The inner nuclear membrane is composed of approximately 80 transmembrane proteins, as suggested by proteomics analysis of mammalian cells (Schirmer and Gerace, 2005). Most of the inner nuclear membrane proteins are involved in interactions between the lamina, chromatin, and inner nuclear membrane (Zastrow *et al.*, 2004). A new class of inner nuclear membrane proteins, called the SUN domain proteins (Sad1/UNC-84 homology), has been proposed to provide a link between the cytoplasm and nuclear interior as they can bind to nesprins as well as lamins (Crisp *et al.*, 2006; Haque *et al.*, 2006; Padmakumar *et al.*, 2005; Tzur *et al.*, 2006). The NPCs are large macromolecular assemblies composed of about 30 proteins collectively termed nucleoporins, and their role in basic nucleocytoplasmic transport processes has been well documented (Hetzer *et al.*, 2005). Studies suggest that NPCs can undergo dynamic changes in structure and composition to regulate transport (Tran and Wente, 2006).

The nuclear lamina was first identified by electron microscopy as a fibrous network underlying the inner nuclear membrane in vertebrate cells (Fawcett, 1966). The major components of the nuclear lamina are a group of nuclear proteins termed the lamins, which belong to the intermediate filament (IF) superfamily of proteins (Goldman *et al.*, 2002; Stuurman *et al.*, 1998). The lamina is considered to be an important determinant of interphase nuclear architecture as it plays an essential role in maintaining nuclear integrity and provides anchoring sites for chromatin (Fig. 4.1). Increasing evidence suggests that lamins are involved in spatial organization of various nuclear functions and can interact with proteins that function in diverse cellular pathways (Broers *et al.*, 2006; Gruenbaum *et al.*, 2005; Worman and Courvalin, 2005). More than 200 mutations in the human lamin A gene (*LMNA*) have been linked to at least 10 highly degenerative, heritable disorders that affect primarily muscle, adipose, or neuronal tissues, and also cause premature aging syndromes (Capell and Collins, 2006). Reports have identified mutations in the lamin B1 and lamin B2 genes that are associated with heritable diseases (Hegele *et al.*, 2006; Padiath *et al.*, 2006).

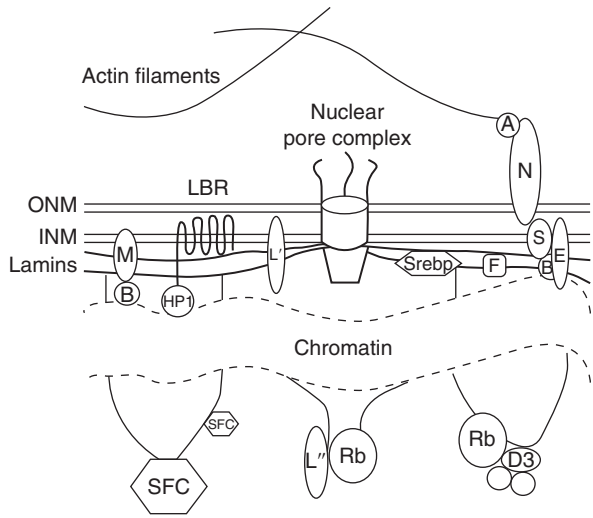

Figure 4.1 Nuclear organization of lamins and associated proteins. The nuclear lamins form a network beneath the inner nuclear membrane (INM), where they interact with INM proteins such as emerin (E), MAN1 (M), lamin B receptor (LBR) and LAP2β (L′), and sequester transcription factors such as Fos (F) and SREBP1. The outer nuclear membrane (ONM) harbors the nesprins (N) that, together with SUN domain proteins (S), bridge the lamina with the actin cytoskeleton through actin-binding proteins (A). Chromatin-binding proteins such as BAF (B) and HP1 interact with the lamina as well as INM proteins. Lamins have also been detected in the nuclear interior in association with pRb and LAP2α (L″), pRb and cyclin D3 (D3) multimeric complexes, and splicing factor compartments (SFCs).

Although the exact mechanisms by which lamin mutations result in disease are not yet understood, current research in this area has given valuable clues about additional functional roles of lamins, especially in specific signaling pathways. This review summarizes the structure and organization of lamins, the genetic disorders associated with lamins, and the roles of lamins in nuclear organization and cellular signaling pathways, with emphasis on more recent findings.

2. Structure and Organization of Lamins

2.1. Genes and expression of lamin isoforms

Two major kinds of lamins are present in animal cells: B-type lamins (B1 and B2), which are found in nearly all somatic cells, and A-type lamins (A and C), which have been detected primarily in differentiated cells. The A- and B-type lamins range in size from 60 to 70 kDa; they differ in solubility properties, expression patterns, and localization during mitosis (Goldman *et al.*, 2002; Stuurman *et al.*, 1998). Lamins A and C (henceforth called lamin A/C) are alternatively spliced products of the lamin A gene, *LMNA*, whereas lamins B1 and B2 are encoded by two separate genes, *LMNB1* and *LMNB2*. Additional splice variants are germ cell-specific lamins C2 and B3, which are encoded by *LMNA* and *LMNB2*, respectively, and a minor somatic cell isoform of lamin A termed lamin AΔ10. *Drosophila melanogaster* has two lamin genes: the lamin Dm_0 gene (*lamDm*$_0$), which is expressed in most cells, and the lamin C gene (*lamC*), the expression of which is developmentally regulated. *Caenorhabditis elegans* has only one lamin gene, *lmn-1*, which is expressed in all cells except for mature sperm. Genome sequence analysis of yeast and *Arabidopsis* indicates that these species do not have lamins and, although functional homologs might exist, these have not yet been definitively identified. Thus lamins appear to have evolved in animal cells.

LMNA has been mapped to the locus 1q21.2-q21.3 in the human genome, whereas *LMNB1* and *LMNB2* are located at loci 5q23.3-q31.1 and 19p13.3, respectively (Biamonti *et al.*, 1992; Wydner *et al.*, 1996). Elucidation of the genomic organization of human *LMNA* has established that it contains 12 exons and 11 introns spread over approximately 24 kb of contiguous genomic DNA (Lin and Worman, 1993) (Fig. 4.2). The first intron is about 16 kb in length, the second intron is about 2 kb, and the remaining introns are less than 1 kb in length. Both lamins A and C share the first nine exons. The alternatively used 3′ splice site for the generation of lamin A and lamin C transcripts lies in exon 10 after amino acid position 566. The codons for amino acids 567–572, which are unique for lamin C, occur immediately after the codon for amino acid 566, and are followed by

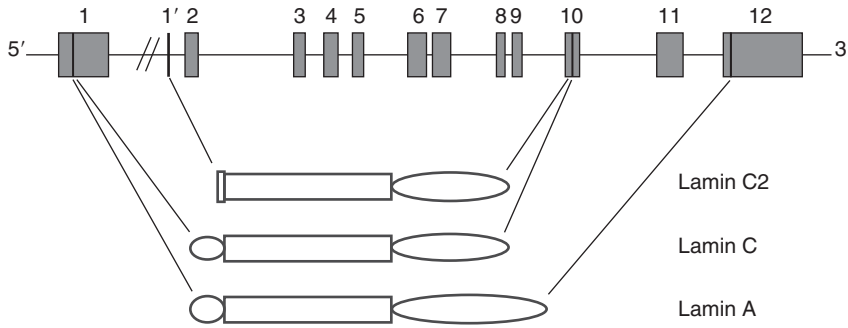

Figure 4.2 Genomic organization of *LMNA*. *LMNA* is composed of 12 exons spread over about 24 kb of DNA and harbors a large first intron of about 16 kb (not drawn to scale). Lamins A and C share the first nine exons whereas the 3′ alternative splice site for lamin C lies in exon 10. Lamin C2 is a 5′ variant of lamin C that has a unique first exon that resides in the first intron of *LMNA*.

a stop codon and a polyadenylation signal in exon 10 to generate lamin C mRNA. Prelamin A mRNA is generated by a lamin A-specific alternative splicing event wherein the 3′ end of codon 566 is joined to the 5′ end of exon 11. Exon 12 has the stop codon and the polyadenylation sequence for lamin A. The sequence of germ cell-specific lamin C2 is similar to that of lamin C, except for a separate exon 1 that encodes the unique N-terminal hexapeptide sequence of lamin C2 and resides within intron 1 of the *LMNA* gene (Nakajima and Abe, 1995). Another alternative splice product of the *LMNA* gene, lamin AΔ10, completely lacks exon 10 and is similar to lamin A except for an internal deletion of 30 amino acids encoded by exon 10 (Machiels *et al.*, 1996). The human lamin B1 gene, *LMNB1*, consists of 11 exons and 10 introns and spans about 45 kb of genomic DNA (Lin and Worman, 1995). The reannotated human lamin B2 gene contains 12 exons and 11 introns (Hegele *et al.*, 2006). The lamin B2 gene encodes a germ cell-specific variant termed lamin B3 (Furukawa and Hotta, 1993). Analysis of about 2 kb of the 5′ flanking sequence of the lamin A/C promoter has revealed the importance of Sp1, Sp3, activating protein 1 (AP-1), and cyclic AMP-responsive element-binding protein (CREB)-binding protein in promoter activation, as well as the presence of a retinoic acid-responsive element (Muralikrishna and Parnaik, 2001; Okumura *et al.*, 2000; Ramaiah and Parnaik, 2006; Tiwari *et al.*, 1998). Although this 5′ segment does not harbor binding sites for tissue-specific regulatory factors, the first intron has been shown to contain motifs that mediate cell type-specific interactions with transcription factors (Arora *et al.*, 2004).

The expression patterns of lamins during development have been studied extensively in vertebrate species such as *Xenopus*, chick, mouse, and human (Goldman *et al.*, 2002; Stuurman *et al.*, 1998). The A-type lamins

have been detected in differentiated cells of most lineages whereas the B-type lamins are present in all nucleated somatic cells. Although lamin A/C has not been detected in embryonic cells of several species (Constantinescu *et al.*, 2005; Riemer *et al.*, 1995; Röber *et al.*, 1989) two reports suggest that there may be species-specific differences in the embryonic expression of lamin A/C. In early embryos of bovine and porcine origin, lamin A/C is detectable up to the eight-cell stage although its levels decline in later embryonic stages (Foster *et al.*, 2007; Hall *et al.*, 2005). The minor variant lamin AΔ10 has been detected in cell lines derived from colon, lung, and breast carcinomas (Machiels *et al.*, 1996). Certain cell types of the hematopoietic and neuronal cell lineages do not express detectable amounts of lamin A/C. Lamin A/C levels are also low or undetectable in a number of malignant tissues and transformed cell lines (Prokocimer *et al.*, 2006). The tissue-specific and temporal regulation of lamin A/C expression has provided the earliest clues for a cell type-specific role for A-type lamins.

2.2. Lamin structure, assembly, and dynamics

Lamins have been classified as type V IF family proteins, and are characterized by a tripartite structure consisting of a central α-helical rod domain flanked by nonhelical N-terminal "head" and C-terminal "tail" domains (Stuurman *et al.*, 1998) (Fig. 4.3). The highest homology amongst lamins has been found within two short segments at either ends of the α-helical rod domain.

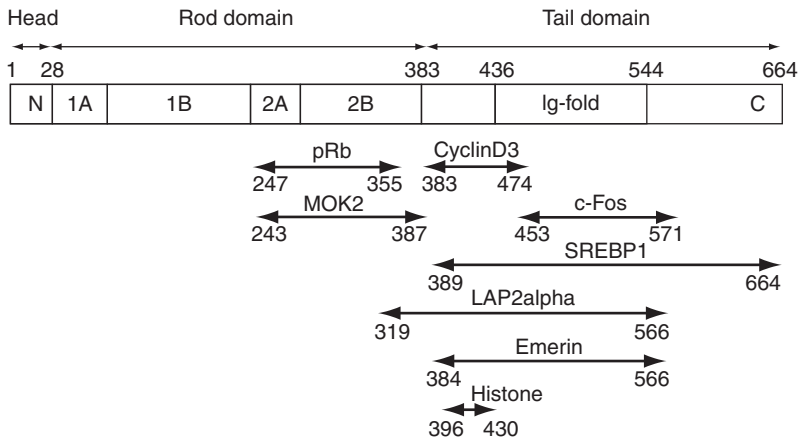

Figure 4.3 Lamin A protein domains and specific protein-binding regions. The major domains in prelamin A (amino acid residues 1–664) are the rod domain composed of α-helical segments 1A, 1B, 2A, and 2B, which are flanked by a short head domain and a tail domain that harbors an immunoglobulin (Ig) fold motif. The binding regions that have been mapped for specific proteins (described in text) are shown.

These two end segments of the rod domain, consisting of about 30 amino acids each, are highly conserved among all other IF proteins. The conserved central rod domain is about 356 amino acids long and consists of four distinct α-helical coils, namely, 1A, 1B, 2A, and 2B. Coil 1B has a 42-amino acid segment composed of six heptad repeats which is also found in invertebrate cytoplasmic IF proteins but not in vertebrate cytoplasmic IF proteins. On the basis of this observation it has been suggested that vertebrate cytoplasmic IF proteins have evolved from lamins. The four α-helical domains are separated by linker segments, namely, L1, L12, and L2, which have been predicted to be α-helical. Linker L1 also maintains the heptad repeat pattern and therefore, together with segments 1A and 1B, it forms a continuous coiled coil. A nuclear localization signal sequence is located after the C-terminal end of the rod domain at residues 417–422. The N-terminal head domain is relatively smaller than the C-terminal tail domain. Both of these domains are of variable lengths in different lamins and play an important role in lamin assembly. The central rod domain drives the interaction between two lamin proteins to form a coiled-coil dimer, the basic structural unit of lamin assembly. The head-to-tail associations between two lamin dimers lead to the formation of protofilaments, which have the propensity to associate laterally in different configurations such as parallel, staggered, or half-staggered to give rise to the 10-nm lamin filament. The head-to-tail linear association of two lamin dimers involves an overlap of the C-terminal part of coil 2B and the N-terminal part of coil 1A. Structural analysis of the lamin coil 2B fragment has revealed potential interactions within the overlapping region involving coil 2B and coil 1A (Strelkov *et al.*, 2004). Although the formation of stable 10-nm filaments has not been observed *in vitro* with vertebrate lamins, which form paracrystals at higher concentrations, *C. elegans* lamin (Ce-lamin) can assemble rapidly into 10-nm filaments *in vitro* (Foeger *et al.*, 2006; Karabinos *et al.*, 2003). It has been suggested that higher order lamin assembly might require interactions with other molecules *in vivo*.

The three-dimensional crystal structure of the lamin A/C globular tail domain, which is 116 residues long, has revealed a compact, well-defined structure composed of nine β strands that form a β sandwich (Dhe-Paganon *et al.*, 2002; Krimm *et al.*, 2002). This structure is referred to as the immunoglobulin (Ig) domain or fold, and is a protein structural unit common to immunoglobulins, several transcription factors, growth factors, and cytokine receptors; Ig domains serve as structural scaffolds or may mediate specific intermolecular interactions with other proteins, DNA, or phospholipids. The addition of the lamin Ig fragment to *Xenopus* nuclear assembly extracts leads to inhibition of chromatin condensation, nuclear assembly, and growth due to impairment of lamin–lamin interactions (Shumaker *et al.*, 2005). Most of the disease-causing mutations in the rod domain affect lamin assembly and cause increased mobility of lamins in live cells (Broers *et al.*, 2005; Gilchrist *et al.*, 2004). Some of the mutations in the

C-terminal domain affect binding to emerin although the mutant proteins correctly assemble into the nuclear rim. The R482W mutation does not affect filament stability (Gilchrist *et al.*, 2004), and this is consistent with predictions from the crystal structure of the C-terminal globular domain (Dhe-Paganon *et al.*, 2002).

The C-termini of lamins A, B1, and B2 bear a CaaX motif (C, cysteine; a, aliphatic; X, any amino acid) that is posttranslationally modified by cysteine farnesylation followed by proteolytic cleavage of the last three amino acids (aaX) and methyl esterification of the carboxyl group of the farnesylated cysteine residue. Farnesylation appears to be required for increasing the hydrophobicity of the C terminus to allow targeting of lamins to the inner surface of the nuclear envelope. After nuclear envelope localization, the C-terminal 15 residues of prelamin A, including the farnesylated cysteine, are cleaved off by endoproteolysis to form mature lamin A, as shown in Fig. 4.4. The zinc metalloprotease ZMPSTE24 can catalyze both of these proteolytic reactions (Bergo *et al.*, 2002; Pendás *et al.*, 2002). Lamin C lacks the CaaX box and may require the presence of other lamins such as A and B1 for proper assembly into the nuclear lamina. Lamin C2, which also lacks the

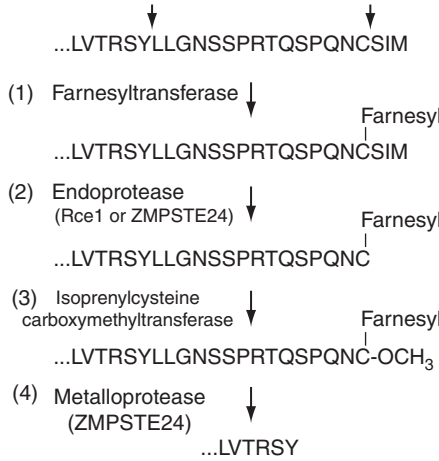

Figure 4.4 Posttranslational processing of the C terminus of prelamin A. Prelamin A bears a CaaX motif (CSIM), which is posttranslationally modified for incorporation into the nuclear envelope in the following steps: (1) A farnesyl group is added to the cysteine residue by the enzyme farnesyl transferase; (2) the terminal three amino acids (SIM) are then cleaved by the ZMPSTE24 or Rce1 endoprotease; (3) the terminal farnesylated cysteine undergoes carboxymethylation; and (4) the terminal 15 amino acids are subsequently removed by a second cleavage by ZMPSTE24. Step 1 can be blocked by farnesyl transferase inhibitors. Steps 2 through 4 are blocked by ZMPSTE24 deficiency, and step (4) is prevented in HGPS cells by removal of the ZMPSTE24 cleavage site.

CaaX motif, is posttranslationally modified at its unique N terminus by myristoylation on the first glycine residue, which confers the hydrophobicity required for nuclear envelope localization (Alsheimer *et al.*, 2000).

In addition to their typical localization at the nuclear periphery, lamins have also been detected in the interior of the nucleus in the form of foci or a diffuse network (Bridger *et al.*, 1993; Goldman *et al.*, 1992; Hozák *et al.*, 1995; Jagatheesan *et al.*, 1999; Moir *et al.*, 1994; Muralikrishna *et al.*, 2004). Some of these intranuclear lamin structures have been implicated in establishing patterns of DNA replication sites and in organizing transcription. DNA replication initiation foci have been shown to colocalize with internal lamin A/C structures (Kennedy *et al.*, 2000; Moir *et al.*, 1994). Parnaik and coworkers have described internal lamin foci or speckles that colocalize with RNA-splicing factors in splicing factor compartments (Jagatheesan *et al.*, 1999). In addition to a typical nuclear rim localization, green fluorescent protein (GFP)-tagged A-type lamins are also localized in an extensive network of intranuclear and transnuclear tubular structures in interphase cells; some of these internal structures contain membrane lipids and are thought to be nuclear envelope invaginations (Broers *et al.*, 1999; Fricker *et al.*, 1997; Moir *et al.*, 2000a). It is not clear to what extent the formation of these structures might be due to overexpression of GFP–lamins, as only a few of these structures are occasionally observed in untransfected cells. Interphase cells expressing GFP-tagged lamin A or B1 also display a veil of nucleoplasmic fluorescence, which represents stable lamin-containing structures (Moir *et al.*, 2000a). A more dynamic internal lamin component is enriched in the G_1 phase of the cell cycle (Broers *et al.*, 1999; Moir *et al.*, 2000a; Muralikrishna *et al.*, 2004). Lamins are dispersed at the onset of mitosis, as a consequence of phosphorylation of essential serine residues on either end of the rod domain of lamin by Cdk1 kinase, which results in depolymerization of the lamina into dimers and tetramers. The lamina is reassembled toward late telophase and in the early G_1 phase of the cell cycle (Gant and Wilson, 1997).

2.3. Lamin-binding proteins

Several inner nuclear membrane proteins have been shown to interact directly with nuclear lamins and to remain closely associated with the nuclear lamina after extraction of nuclei with nonionic detergents, nucleases, and high salt buffers. Various regulatory molecules also bind to lamins and these interactions can influence gene expression in distinct pathways.

2.3.1. Nuclear membrane proteins

The majority of proteins associated with the inner nuclear membrane interact with lamins and/or chromatin. The best characterized lamin-binding proteins are lamin B receptor (LBR), lamina-associated polypeptides (LAPs), and

emerin (Gerace and Foisner, 1994). LBR was the first transmembrane protein identified to interact with B-type lamins. LBR has a predicted eight-transmembrane segment at the C-terminal domain and an N-terminal nucleoplasmic domain that interacts with heterochromatin protein 1 (HP1), chromatin, and B-type lamins *in vitro*. LAP1A, LAP1B, and LAP1C are alternatively spliced products of the *LAP1* gene. LAP1A and LAP1B have been shown to interact with lamin A, lamin C, and lamin B1. LAP1C is anchored to the nuclear envelope as part of a multimeric complex that includes LAP1A and B-type lamins but not A-type lamins. LAP2 proteins are expressed as six isoforms, namely, α, β, γ, δ, ϵ, and ξ and are alternatively spliced products of a single gene. All these isoforms except LAP2α possess a conserved membrane-spanning domain and are classified as type II integral membrane proteins. LAP2β binds specifically to lamin B1, chromatin, and barrier-to-autointegration factor (BAF) (Shumaker *et al.*, 2001). LAP2α lacks the transmembrane domain but has a long nuclear localization signal (NLS)-containing nucleoplasmic domain. It is the only member of the LAP family that is not a membrane protein and does not bind to the peripheral lamina. LAP2α is distributed in the nucleoplasm, where it associates with chromatin and intranuclear lamin A (Dechat *et al.*, 2000; Markiewicz *et al.*, 2002). LAP2α also binds to retinoblastoma protein (pRb) and this association is important for cell cycle regulation (Markiewicz *et al.*, 2002; Pekovic *et al.*, 2007). A newly identified interacting protein of LAP2α, termed LINT-25, is likely to be involved in cell cycle exit (Naetar *et al.*, 2007).

Emerin, LAP2, and another envelope protein, MAN1, possess a 40-residue folded motif called the LEM domain (derived from *L*AP, *e*merin, *M*AN1) that binds directly to BAF, a conserved DNA-binding protein that is involved in higher order chromatin structure and is important for nuclear assembly (Zastrow *et al.*, 2004). Emerin interacts specifically with both A-type lamins and BAF at the nuclear periphery (Lee *et al.*, 2001). Emerin and lamin C depend on lamin A for nuclear envelope localization as in the absence of lamin A/C or on expression of dominant negative lamin mutants, emerin is mislocalized to the endoplasmic reticulum (ER) (Sullivan *et al.*, 1999; Vaughan *et al.*, 2001). Emerin has been reported to bind to β-catenin and to regulate its accumulation in the nucleus (Markiewicz *et al.*, 2006). MAN1 (also termed LEMD3) is retained in the inner nuclear membrane via interactions between its N-terminal nucleoplasmic domain and emerin or lamins (Paulin-Levasseur *et al.*, 1996; Lin *et al.*, 2000; Mansharamani and Wilson, 2005). MAN1 mediates signaling through transcriptional regulators termed Smads, as discussed in Section 5.4. An MAN1-related protein called LEM2 is also associated with lamins A/C, which help to target it to the inner nuclear membrane (Brachner *et al.*, 2005). The mobilities of emerin and MAN1 are increased in cells lacking lamin A/C, consistent with a role for lamin A/C in retaining these proteins at the inner nuclear membrane (Östlund *et al.*, 2006). Other inner nuclear

membrane proteins such as Young Arrest and otefin, which are involved in *Drosophila* oocyte development, have been shown to interact with *Drosophila* lamin Dm_0 (Goldberg *et al.*, 1998).

Another family of nuclear membrane proteins that interact directly with lamin A and emerin consists of the nesprins, which have multiple spectrin repeat motifs at their N termini (Zhang *et al.*, 2001). These motifs serve as sites for protein–protein interaction, actin and microtubule cross-linking, molecular scaffolding, and stabilization. The conserved C-terminal domain of nesprins, which has been termed the KASH domain (for *K*larsicht, *A*NC-1 and *S*yne *h*omology), contains a single transmembrane segment. In mammals, two genes encode multiple isoforms of the two proteins: nesprin-1 (also referred to as enaptin/syne-1/myne-1/CPG2) and nesprin-2 (or syne-2/myne-2/NUANCE). The *C. elegans* protein ANC-1 is an ortholog of the nesprins (Starr and Han, 2002). The nesprin isoforms exhibit tissue-specific expression patterns due to alternative splicing (Mislow *et al.*, 2002a; Zhen *et al.*, 2002). The larger isoforms of nesprin-1 and nesprin-2 are located in the outer nuclear membrane and contain an N-terminal calponin homology domain, which is able to bind to actin. Some of the smaller nesprin isoforms are localized in the inner nuclear membrane (Zhang *et al.*, 2005). Nesprin-1 and nesprin-2 bind directly to A-type lamins and emerin and are dependent on lamin A for proper localization in the nuclear envelope (Libotte *et al.*, 2005; Mislow *et al.*, 2002b). Myne-1, which is expressed predominantly in cardiac, skeletal, and smooth muscles, also interacts directly with lamins A and C (Mislow *et al.*, 2002a). The *Drosophila* muscle protein MSP-300 is a nesprin homolog (Zhang *et al.*, 2002). Nesprins have also been detected in multiple cytoplasmic compartments and at the actin cytoskeleton (Zhang *et al.*, 2005). The outer membrane protein nesprin-3 is associated with cytoplasmic IFs via plectin proteins (Wilhelmsen *et al.*, 2005).

The SUN domain proteins contain a conserved motif of about 120 amino acids that was initially discovered in *C. elegans* UNC-84 protein and *Schizosaccharomyces pombe* Sad1 protein (Malone *et al.*, 1999). Most SUN domain proteins contain multiple transmembrane domains and localize to the inner nuclear membrane. The N-terminal domains of SUN-1 and SUN-2 are located in the nucleoplasm and bind directly to A-type lamins; the C-terminal domains are localized in the lumen of the nuclear envelope, where they interact with nesprins (Crisp *et al.*, 2006; Haque *et al.*, 2006; Padmakumar *et al.*, 2005). The nesprins and SUN domain proteins have been proposed to bridge the nuclear envelope and provide connectivity between the nucleoplasm and cytoskeleton, raising interesting possibilities for transduction of cellular signals (Crisp *et al.*, 2006; Haque *et al.*, 2006; Hutchison and Worman, 2004; Padmakumar *et al.*, 2005).

2.3.2. Gene-regulatory proteins

Earlier *in vitro* analysis has shown that lamins A/C can bind to DNA (Luderus *et al.*, 1994), chromatin (Glass *et al.*, 1993), and core histones (Taniura *et al.*, 1995). Lamin A associates with the chromatin-binding factor BAF, and this interaction has been proposed to help in interlinking of chromatin, lamins, and LEM domain proteins (Zastrow *et al.*, 2004). There is substantial evidence that lamin A/C associates with specific gene regulatory factors and thereby modulates their activities. The active hypophosphorylated form of the retinoblastoma protein (pRb) can bind to the α-helical rod domain of the A-type lamins (Mancini *et al.*, 1994; Ozaki *et al.*, 1994). pRb is a tumor suppressor protein involved in regulation of the cell cycle and apoptosis as well as in muscle and adipocyte differentiation. In the early G_1 phase, hypophosphorylated pRb binds to the transcription factor E2F, thus blocking E2F-dependent expression of S-phase genes and entry into S phase. Hypophosphorylated Rb can also interact with LAP2α, and LAP2α lamin A/C complexes are able to anchor pRb to the nuclear envelope (Markiewicz *et al.*, 2002). Binding interactions between lamin A/C, pRb, and cyclin D3 have been proposed to play an important role in muscle differentiation (Mariappan and Parnaik, 2005; Mariappan *et al.*, 2007). A-type lamins are also involved in regulating the function of pRb by promoting its proper subnuclear localization and preventing its proteasomal degradation (Johnson *et al.*, 2004). A transcriptional repressor of E2F-mediated gene expression, termed germ cell-less (GCL), which is required to establish the germ cell lineage during development in *Drosophila*, has been shown to form a stable ternary complex with emerin and lamin A (Holaska *et al.*, 2003) and to interact with LAP2β (Nili *et al.*, 2001). Another transcriptional repressor reported to bind to lamin A/C is mouse Kruppel-like factor 2 (MOK2). MOK2 represses transcription of the interphotoreceptor retinoid-binding protein (IRBP) gene by competing with a cone–rod homeobox protein (Crx) for its DNA-binding site on the IRBP promoter. The interaction of MOK2 with lamin A/C and the nuclear matrix may facilitate its repressor function (Dreuillet *et al.*, 2002). BAF also binds directly to several homeodomain transcription activators, including Crx, and represses Crx-dependent gene expression in retinal cells (Wang *et al.*, 2002). Association of the transcription factor Oct-1 with lamin B has been correlated with its repressor activity, as dissociation of Oct-1 from the nuclear periphery coincides with the activation of collagenase expression when cells approach senescence (Imai *et al.*, 1997). Lamin A has been reported to bind to c-Fos and sequester it at the nuclear periphery, leading to repression of AP-1 transcriptional activity (Ivorra *et al.*, 2006). A schematic of the binding sites on lamin A that have been identified for various regulatory factors is given in Fig. 4.3.

An adipocyte differentiation factor, sterol response element-binding protein 1 (SREBP1), has been shown to interact directly with lamin A;

both isoforms, SREBP1a and SREBP1c, are capable of interacting with lamins by binding to the Ig fold of the lamin A/C tail domain (Lloyd *et al.*, 2002). SREBP proteins are basic helix–loop–helix leucine zipper transcription factors and regulate the genes required for cholesterol biogenesis and lipogenesis and also promote adipocyte differentiation. Protein kinase Cα (PKCα), a serine/threonine kinase activated by various signaling molecules such as diacylglycerol and 12(*S*)-hydroeicosatetraenoic acid [12(*S*)-HETE], binds to the C-terminal domain of lamin A/C (Martelli *et al.*, 2002), implying that the lamina might be involved in regulating lipid-induced PKCα signaling.

Heat shock proteins such as Hsp70 and a small heat shock protein, Hsp26, associate with nuclear lamins (Willsie and Clegg, 2002). Other Hsps such as αB-crystallin and Hsp25 have been observed to colocalize with splicing factor compartments and with intranuclear lamin A/C speckles in heat-stressed cells (Adhikari *et al.*, 2004). It has been proposed that αB-crystallin and Hsp25 might associate with and stabilize intranuclear lamin A/C under heat stress conditions in a differentiation-specific manner in skeletal muscle cells (Adhikari *et al.*, 2004). Binding interactions between small heat shock proteins and IF proteins are important in the physiological context as mutations in the small heat shock protein Hsp27 have been associated with the inherited neuromuscular disease Charcot-Marie-Tooth disease type 2F and with distal hereditary motor neuropathies, which affect neurofilament assembly (Evgrafov *et al.*, 2004), and a mutation in αB-crystallin (R120G) causes a desmin-related cardiomyopathy (Vicart *et al.*, 1998). Interestingly, fibroblasts from patients with Hutchinson-Gilford progeria syndrome (HGPS) due to a G608G mutation in *LMNA* (see Section 3.1.4) are hypersensitive to heat shock (Paradisi *et al.*, 2005).

3. Genetic Diseases Associated with Mutations in Lamins and Nuclear Envelope Proteins

3.1. Diseases caused by mutations in lamin A

The major class of inherited diseases caused by mutations in *LMNA* is termed primary laminopathies. A second class of related diseases is caused by mutations in the *ZMPSTE24* gene, which hinder the normal posttranslational processing of prelamin A, and have been designated as secondary laminopathies. The tissue-specific laminopathies affect striated muscles, adipose tissue, and peripheral nerves, whereas the premature aging syndromes afflict several tissues (summarized in Table 4.1). Certain cases of overlapping symptoms have also been described.

Table 4.1 Range of Genetic Diseases Caused by Mutations in *LMNA* and *ZMPSTE24*

Disease	Inheritance	Mutations	Clinical symptoms
Emery-Dreifuss muscular dystrophy	AD (rarely AR)	Missense, all exons (>100 mutations)	Muscle contractures, wasting of skeletal muscle; cardiomyopathy with conduction defects
Limb girdle muscular dystrophy 1B	AD	Missense	Slowly progressive wasting of shoulder and pelvic muscles, cardiac disturbances
	AR	Nonsense (Y259X)	Lethal
Dilated cardiomyopathy	AD	Missense, exons 1 and 3	Ventricular dilation, systolic dysfunction, conduction defects
Familial partial lipodystrophy	AD	Missense, exons 8 and 11 (mostly at R482)	Loss of fat tissue from extremities, accumulation in neck and face, insulin-resistant diabetes, hyperlipidemia, atherosclerosis
Generalized lipoatrophy	AD	R133L, T10I	General lipodystrophy, insulin-resistant diabetes, progeroid features
Charcot-Marie-Tooth disorder 2B	AR	R298C	Axonal degeneration, sensory impairment
Hutchinson-Gilford progeria	*De novo*	GGC to GGT in codon 608, forms progerin	Features of premature aging, early mortality (by 12 years)

Table 4.1 (*continued*)

Disease	Inheritance	Mutations	Clinical symptoms
Atypical Werner's syndrome	AD	A57P, R133L, L140R	Features of premature aging, affects young adults
Mandibuloacral dysplasia A	AR	R527H	Skull and face anomalies, skeletal abnormalities, partial lipodystrophy, premature aging symptoms
Mandibuloacral dysplasia B	AR	*ZMPSTE24* mutations	Generalized lipodystrophy
Restrictive dermopathy	*De novo* for *LMNA*, AR for *ZMPSTE24*	Splicing defects, truncated proteins	Intrauterine growth retardation, tight and rigid skin, neonatal mortality

AD, autosomal dominant; AR, autosomal recessive.

3.1.1. Striated muscle diseases

The clinical condition termed Emery-Dreifuss muscular dystrophy (EDMD) was first described by Alan Emery and Fritz Dreifuss in the 1960s (Emery and Dreifuss, 1966). This condition is marked by contractures of the elbows, Achilles tendons, and posterior neck; slow progressive muscle wasting; and dilated cardiomyopathy with atrioventricular conduction block. The gene responsible for X-linked EDMD was identified by a positional cloning approach and named ''emerin'' (Bione *et al.*, 1994). Emerin was shown to be deficient in cells of patients suffering from EDMD (Manilal *et al.*, 1996; Nagano *et al.*, 1996). Subsequently, an autosomal dominant form of EDMD has been attributed to mutations in *LMNA* (Bonne *et al.*, 1999; Brown *et al.*, 2001). About 60–70% of cases due to *LMNA* mutations show involvement of striated muscles. Most of the mutations are missense mutations and a few are small deletions or nonsense mutations; mutations are found in all the exons of the gene. Both familial and sporadic mutations have been identified. For the same mutation, there is

considerable variability in the range of symptoms observed in different families as well as within a family; some patients show the full clinical symptoms of EDMD whereas others show only cardiac symptoms (Bonne *et al.*, 2000). Hence the occurrence of modifier genes cannot be ruled out. A rare case of autosomal recessive EDMD was caused by the inheritance of two different mutant *LMNA* alleles (Raffaele Di Barletta *et al.*, 2000). Another case of autosomal recessive EDMD has been documented to be due to homozygosity for an H222Y mutation; the parents of the individual did not show skeletal or cardiac dysfunction (Sanna *et al.*, 2003).

Familial cases of dilated cardiomyopathy (DCM) have been associated with defects in more than 20 genes. Autosomal dominant mutations in *LMNA* are the most common cause of DCM (Fatkin *et al.*, 1999) and lead to a particularly severe form of the disease, which has been subgrouped as dilated cardiomyopathy type 1A (CMD1A). DCM is a progressive disease that is characterized by ventricular dilation and systolic dysfunction. In patients with *LMNA* mutations, DCM is usually accompanied by conduction defects and may include skeletal muscle involvement. Because of the risk of sudden death among such patients, an effective therapeutic intervention is the use of an implantable cardioverter–defibrillator to treat possibly lethal ventricular arrhythmias (Meune *et al.*, 2006). Missense mutations and splicing defects in *LMNA* have also been linked to autosomal dominant limb girdle muscular dystrophy type 1B (LGMD1B) (Muchir *et al.*, 2000). LGMD1B is a slowly progressing disease characterized by weakness and wasting of shoulder and pelvic muscles due to necrosis, and is accompanied by cardiac conduction defects in several patients. A lethal phenotype has been reported for a newborn child (from an LGMD1B family) who displayed homozygosity for a Y259X nonsense mutation (Muchir *et al.*, 2003).

3.1.2. Lipodystrophies and related disorders

Mutations in *LMNA* have been linked to Dunnigan-type familial partial lipodystrophy (FPLD) by several groups (Cao and Hegele, 2000; Shackleton *et al.*, 2000; Speckman *et al.*, 2000). FPLD is an autosomal dominant disorder characterized by loss of fat tissue from the extremities and excess fat accumulation on the face and neck, beginning at puberty. This is accompanied by insulin-resistant diabetes, hyperlipidemia, and atherosclerotic vascular disease. Approximately 90% of the mutations in FPLD are located to exon 8, with substitutions at arginine at amino acid position 482 being found in 75% of cases. Cardiomyopathy and muscle weakness have been reported in FPLD patients with mutations in exon 1 (Garg *et al.*, 2002; van der Kooi *et al.*, 2002) but not in FPLD patients with mutations in exons 8 or 11. A particularly severe case of FPLD has been attributed to a novel mutation at the intron 8 consensus splice donor site, which leads to a prematurely terminated lamin A isoform (Morel *et al.*, 2006). Generalized lipoatrophy has been associated with an R133L *LMNA* mutation and is accompanied

by insulin-resistant diabetes and cardiomyopathy (Caux *et al.*, 2003). A patient with Seip syndrome showing generalized lipodystrophy together with features of progeroid syndromes including short stature, scleroderma-like skin, and early graying of hair has been reported to harbor a T10I *LMNA* mutation (Csoka *et al.*, 2004a).

3.1.3. Peripheral neuropathy

The Charcot-Marie-Tooth disorders (CMTs) are a group of neuromuscular diseases that show considerable clinical and genetic heterogeneity. An autosomal recessive mutation at R298C of *LMNA* gives rise to CMT type 2B, which is an axonal neuropathy characterized by peripheral loss of large myelinated fibers and axonal degeneration (De Sandre-Giovannoli *et al.*, 2002). Patients showed sensory impairment with some reduction in motor nerve conduction velocity. However, heterozygous carriers did not exhibit clinical signs of neuropathy. Cases have also been reported with the dominant *LMNA* mutations E33D and R571C, which result in overlapping symptoms of muscular dystrophy and neuropathy, with cardiac disease (Goizet *et al.*, 2004; Benedetti *et al.*, 2005).

3.1.4. Premature aging syndromes

The most dramatic effects of mutations in *LMNA* have been observed in the premature aging disorder HGPS (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003). HGPS is a very rare disorder (affecting about 1 in 1 million) that leads to early mortality, usually in the second decade of life. HGPS is an autosomal dominant condition that is characterized by short stature, early thinning of skin, loss of subcutaneous fat, premature atherosclerosis, and cardiac failure leading to death. The majority of cases are due to a *de novo* missense mutation (GGC to GGT) in exon 11 that does not cause an amino acid change (G608G), but leads to creation of an abnormal splice donor site that results in expression of a truncated prelamin A protein (also termed progerin or lamin AΔ50) with loss of 50 amino acids from the C terminus including the second ZMPSTE24 cleavage site, resulting in a permanently farnesylated C terminus (see Fig. 4.4). Compound heterozygous missense mutations such as R471C/R527C and T528M/M540T, which do not result in expression of truncated prelamin A protein, have also been described in HGPS patients (Cao and Hegele, 2003; Verstraeten *et al.*, 2006). *LMNA* mutations that lead to atypical progeroid syndromes have been reported (Csoka *et al.*, 2004a). Chen *et al.* (2003) have described patients with atypical Werner's syndrome with an early adult-onset phenotype and less severe disease than HGPS, who were found to have missense mutation A57P, R133L, or L140R in *LMNA*. A case of autosomal recessive HGPS has been reported with a K542N mutation in *LMNA* (Plasilova *et al.*, 2004); phenotypes in the family of this patient overlapped with those observed in mandibuloacral dysplasia.

Mandibuloacral dysplasia (MAD) is a rare, autosomal recessive disorder characterized by postnatal growth retardation, skull and facial anomalies, skeletal abnormalities, mottled skin pigmentation, partial or generalized lipodystrophy, and signs of premature aging (Novelli *et al.*, 2002). Most patients with MAD type A, showing partial lipodystrophy, have been reported to have an R527H homozygous mutation in *LMNA*. A patient with an R527C/R471C heterozygous mutation, reported to have atypical progeria, also showed symptoms of MAD (Cao and Hegele, 2003). In addition, MAD type B, characterized by generalized loss of fat involving face, trunk, and extremities, is caused by mutations in ZMPSTE24 protease, which is involved in the processing of prelamin A to lamin A (Agarwal *et al.*, 2003). Metabolic studies have revealed hyperinsulinemia in many subjects of MAD.

Mutations in *LMNA* as well as *ZMPSTE24* are associated with restrictive dermopathy, a rare disorder characterized by intrauterine growth retardation, tight and rigid skin with erosions, facial malformation, bone mineralization defects, and early neonatal mortality (Navarro *et al.*, 2004, 2005; Shackleton *et al.*, 2005). Some of the mutations in *LMNA* or *ZMPSTE24* lead to the creation of premature termination codons, resulting in truncated proteins. One case has been diagnosed with the G608G mutation, identical to that observed in HGPS (Navarro *et al.*, 2004), with phenotypes in between those of HGPS and restrictive dermopathy. Thus HGPS, MAD, and restrictive dermopathy appear to represent a clinical spectrum of related disorders, with the severity depending on the dysfunction of lamin A/C. A case has also been reported with a combination of myopathy and progeria, due to an S143F mutation in *LMNA* (Kirschner *et al.*, 2005).

3.2. Diseases caused by mutations in lamin B

Mutations in the human lamin B genes have been linked to inherited diseases in more recent reports. Missense mutations in the lamin B2 gene have been associated with acquired partial lipodystrophy (APL) in four patients (Hegele *et al.*, 2006). The main features of APL are a gradual loss of subcutaneous fat from the head, neck, upper extremities, and thorax but not from the lower extremities. Duplications of the lamin B1 gene have been identified in patients with adult-onset leukodystrophy (Padiath *et al.*, 2006). This is a progressive neurological disorder characterized by loss of myelin in the CNS. Downregulation of either lamin B1 or B2 is lethal for dividing cultured cells (Harborth *et al.*, 2001) and knockout of the lamin B1 gene in mice results in death in early development (Vergnes *et al.*, 2004). However, distinctive functions for lamin B1 or B2 have not yet been identified.

3.3. Disorders associated with mutations in other nuclear envelope proteins

As mentioned in Section 3.1.1 , mutations in the emerin gene (*EMD*) cause X-linked EDMD (Bione *et al.*, 1994; Emery and Dreifuss, 1966). The majority of mutations in *EMD* are null mutations that are spread throughout the gene. Missense mutations in *EMD* give rise to a milder form of EDMD. Heterozygous mutations in the LBR gene lead to a relatively mild autosomal dominant disorder termed Pelger-Huët anomaly (Hoffmann *et al.*, 2002), which is characterized at the cellular level by abnormal nuclear morphology of blood granulocytes, and is associated with varying levels of developmental delay, epilepsy, and skeletal abnormalities. A recessive disorder called Greenberg skeletal dysplasia is a lethal chondrodystrophy that is caused by homozygous mutations in the LBR gene (Waterham *et al.*, 2003). Mutations in the MAN1 gene (*LEMD3*) cause osteopoikilosis, Buschke-Ollendorff syndrome, and melorheostosis, which are disorders that affect mainly bone and skin tissues (Hellemans *et al.*, 2004). A mutation in the LAP2α gene has been linked to DCM (Taylor *et al.*, 2005). An early-onset autosomal dominant dystonia, which is characterized by involuntary, sustained muscle contractions, is caused by mutations in an ATPase termed torsinA (Ozelius *et al.*, 1997). TorsinA is normally resident in the ER lumen but disease-causing mutant torsinA is mislocalized to the lumen of the nuclear envelope, where it atypically binds to LAP1 (Goodchild and Dauer, 2005). Hence alterations in interactions between inner membrane proteins and specific binding partners may be an underlying cause for certain inherited diseases. As proteomic analysis has indicated that the inner nuclear membrane is composed of about 80 transmembrane proteins (Schirmer and Gerace, 2005), of which several proteins have not yet been characterized in detail, it is possible that more genetic disorders may be attributed to mutations in genes encoding these proteins in future studies.

3.4. Animal models for laminopathies

Valuable models for laminopathies have been generated by gene-targeting studies in mice. A gene knockout in exons 8–11 of the mouse lamin A gene (*Lmna*) leads to the development of muscular dystrophy symptoms resembling EDMD and results in mortality by 2 months of age (Sullivan *et al.*, 1999). The $Lmna^{-/-}$ animals also show loss of white adipose tissue (but not partial lipodystrophy or insulin resistance), cardiac dysfunction (Nikolova *et al.*, 2004), nonmyelinated axons and reduced axon density (De Sandre-Giovannoli *et al.*, 2002), and defects in spermatogenesis (Alsheimer *et al.*, 2004). A mouse knock-in model for EDMD has been obtained by gene targeting of a mouse *Lmna* fragment with an H222P mutation (Arimura *et al.*, 2005), a mutation that had been earlier identified in a family with

autosomal dominant EDMD (Bonne *et al.*, 2000). Homozygous H222P knock-in mice develop skeletal muscle degeneration and cardiac dysfunction by adulthood but do not show symptoms at earlier stages. Genome-wide expression profiling of heart tissue from H222P knock-in mice and emerin knockout mice has revealed a common activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) branch of the mitogen-activated protein kinase pathway in both models (Muchir *et al.*, 2007). Interestingly, mice that lack emerin are overtly normal and do not show a dystrophic phenotype (Melcon *et al.*, 2006). Homozygous mice with knock-in of the N195K mutation, which leads to DCM in humans, show disruption in the organization of cardiomyocytes together with mis-regulation of factors required for normal cardiac development (Mounkes *et al.*, 2005). Heart-specific expression of the EDMD mutation M371K in a transgenic mouse model leads to cardiac dysfunction, abnormal nuclear morphology in cardiac cells, and early death (Wang *et al.*, 2006), which demonstrates that expression of certain mutations even in the presence of wild-type lamin A/C can lead to deleterious effects via dominant mechanisms in mice.

Another useful mouse model is the *Zmpste24*-null mouse, which develops progressive bone disease and muscle weakness, as well as progeria-like features (Bergo *et al.*, 2002; Pendás *et al.*, 2002). Unprocessed prelamin accumulates in the nuclei of these mice, leading to irregular nuclei with herniations and nuclear blebs. A mouse model for progeria has been derived by an unexpected deletion of exon 9 due to splicing defects (Mounkes *et al.*, 2003). A progerin knock-in model has been described in which heterozygous mice exhibit growth retardation and fibroblasts from these mice show aberrant nuclear morphology, whereas homozygous mice have severe osteoporosis and die soon after birth (Yang *et al.*, 2005). A mouse transgenic model bearing the human *LMNA* gene with a G608G mutation shows progressive loss of vascular smooth muscle cells (Varga *et al.*, 2006), which has also been observed in HGPS patients (McClintock *et al.*, 2006).

It may be noted that many of these mouse models show overt disease symptoms only in the homozygous state, unlike the autosomal dominant nature of the human diseases in which only one allele is mutated, or the haploinsufficiency caused by the EDMD Q6X mutation (Bonne *et al.*, 1999). Further, emerin is proteolytically degraded in lamin-deficient fibroblasts from a patient with a Y259X mutation (Muchir *et al.*, 2006), but emerin is not degraded in *Lmna*$^{-/-}$ fibroblasts (Sullivan *et al.*, 1999). Thus all features of the human laminopathies might not be faithfully reproduced by the mouse models. Mouse gene knockout models generally display a high degree of functional redundancy in various signaling pathways, as well as compensatory changes in gene expression. Interestingly, mice that have been targeted to express only lamin C and not lamin A are healthy and exhibit only minimal nuclear abnormalities (Fong *et al.*, 2006a).

Although lamin A and prelamin A appear to be dispensable in the mouse from this study, sufficient information is not yet available on the extent to which the roles of lamin A and C might overlap.

4. Role of Lamins in Nuclear Organization and Cellular Functions

4.1. Nuclear morphology and chromatin organization

As lamins are the major architectural proteins of the nucleus, they have been proposed to play a crucial role in maintenance of nuclear shape and integrity, organization of chromatin and distribution of nuclear pore complexes. This is strongly supported by earlier findings with loss-of-function lamin mutants in *C. elegans* and *Drosophila melanogaster*, in addition to studies with laminopathic cells and *in vitro* binding data with chromatin-associated proteins such as BAF and LAP2α (see Section 2.3.1). In *C. elegans*, which has a single lamin gene, loss of lamin causes aberrant heterochromatin organization, unequal separation of chromosomes, and abnormal distribution of nuclear pore complexes, leading to embryonic lethality (Liu *et al.*, 2000). Mutations in *Drosophila* lamin Dm_0 (a B-type lamin) result in developmental abnormalities in organogenesis, locomotion, tracheal development, and nuclear positioning in the oocyte and eye, as well as aberrant nuclear morphology and clustering of nuclear pore complexes (Guillemin *et al.*, 2001; Lenz-Bohme *et al.*, 1997; Osouda *et al.*, 2005; Patterson *et al.*, 2004). Furthermore, overexpression of lamin Dm_0 leads to highly degenerative phenotypes (Padiath *et al.*, 2006). Mutations in *Drosophila* lamin C, an A-type lamin, are lethal and an R401K mutation (homologous to the R386K mutation that causes EDMD in humans) shows defects in nuclear morphology (Schulze *et al.*, 2005). One study carried out by Parnaik and colleagues indicates that lamin C is essential for tissue-specific development and chromatin organization in *Drosophila* (Gurudatta *et al.*, 2007). B-type lamins from *Xenopus* sperm nuclei interact with the nuclear pore complex protein Nup153 and help to localize it within the nucleoplasmic ring (Smythe *et al.*, 2000). Furthermore, nuclei assembled in *Xenopus* egg extracts in which lamina formation has been disrupted are fragile (Spann *et al.*, 1997).

Severe nuclear abnormalities have been reported in HGPS cells, including lobulation, blebbing, and loss of heterochromatin (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003; Goldman *et al.*, 2004). Although HGPS nuclei show normal resistance to mechanical stress, the lamina in HGPS cells has a reduced ability to rearrange under mechanical stress (Dahl *et al.*, 2006). It has been proposed that the accumulation of farnesylated prelamin A is toxic to cells and leads to pathogenesis (Fong *et al.*, 2004), and this is supported by evidence of improvement of

nuclear morphology by inhibiting abnormal splicing of prelamin A mRNA (Scaffidi and Misteli, 2005), blocking farnesyl transferase activity in cells (Capell *et al.*, 2005; Columbaro *et al.*, 2005; Glynn and Glover, 2005; Mallampalli *et al.*, 2005; Varga *et al.*, 2006; Yang *et al.*, 2005), knocking out the *Zmpste24* gene in a mouse model (Fong *et al.*, 2006b), or reducing the levels of prelamin A by RNA interference assays (Huang *et al.*, 2005). Importantly, it has been reported that a farnesyl transferase inhibitor administered to *Zmpste24*-deficient mice can decrease progeria-like disease symptoms and improve survival (Fong *et al.*, 2006b), raising the possibility of beneficial effects of these drugs in humans. Abnormal nuclear morphology has also been observed in cells from patients with other laminopathies. Fibroblasts from patients with EDMD, LGMD, DCM, and FPLD show abnormal nuclear phenotypes with nuclear blebbing and aberrant lamin foci in up to 20% of cells (Capanni *et al.*, 2003; Favreau *et al.*, 2003; Muchir *et al.*, 2004; Vigouroux *et al.*, 2001). Exogenous expression of several lamin A/C mutants in mouse or human cells causes aberrant nuclear morphology, altered lamina assembly, mislocalization of emerin, and disruption of the endogenous nuclear lamina (Bechert *et al.*, 2003; Favreau *et al.*, 2003; Manju *et al.*, 2006; Östlund *et al.*, 2001; Raharjo *et al.*, 2001; Vigouroux *et al.*, 2001). Aberrant nuclear morphology results in cellular senescence, downregulation of transcription, and apoptosis (Alsheimer *et al.*, 2004; Capanni *et al.*, 2003; Goldman *et al.*, 2004; Lammerding *et al.*, 2004). An interesting observation is that nuclei from old individuals acquire defects that are similar to those seen in cells from HGPS patients, and this has been attributed to accumulation of progerin (Scaffidi and Misteli, 2006). A study with *C. elegans* has shown that there are age-dependent changes in nuclear shape and loss of peripheral heterochromatin in nonneuronal cells, and the rate of these changes is affected by the insulin/IGF-1-like signaling pathway, which plays an important role in the aging process (Haithcock *et al.*, 2005). These findings support the hypothesis that defects in nuclear architecture might be a cause of normal aging.

There is increasing evidence of a role for lamins in the regulation of epigenetic marks in chromatin from studies with laminopathic cells. Loss of heterochromatin in HGPS cells is accompanied by downregulation of trimethylation at Lys-9 of histone H3 (H3K9), which normally marks pericentric constitutive heterochromatin, and reduced association with heterochromatin protein 1α (HP1α) (Columbaro *et al.*, 2005; Shumaker *et al.*, 2006). Furthermore, the inactive X chromosome from a female HGPS patient shows loss of trimethylation at Lys-27 of histone H3 (H3K27), a mark of facultative heterochromatin (Shumaker *et al.*, 2006). Interestingly, changes in facultative heterochromatin were observed in HGPS cells at earlier passages, before alterations in constitutive heterochromatin and also before changes in nuclear shape. Cells from older MAD type A patients also exhibit accumulation of prelamin A and loss of peripheral

heterochromatin, together with mislocalization of HP1β, trimethylated H3K9, and LBR (Filesi *et al.*, 2005).

Studies have implicated the lamina in additional levels of chromatin organization, specifically in the positioning of certain chromosomes. Chromosomes occupy discrete, nonrandom regions or territories within the nucleus, which may vary according to cell type and growth state; moreover, gene-poor chromosomes are generally located at the nuclear periphery whereas gene-rich chromosomes are found more toward the interior (Cremer and Cremer, 2001). It has been hypothesized that the nonrandom positioning of chromosomes may involve interactions between chromatin and nuclear structures. Several studies have shown that association of certain gene loci with the nuclear periphery in mammalian cells has a repressive effect on the transcription of these genes (Chuang *et al.*, 2006; Hewitt *et al.*, 2004; Zink *et al.*, 2004). A requirement for functional lamin A has been observed for the appropriate localization of the human 4q35.2 region implicated in facioscapulohumeral muscular dystrophy (Masny *et al.*, 2004). Studies have given important insights into possible roles for lamins in chromosome positioning. In one study, the absence of lamin B1 or lack of its C terminus and processing was observed to affect specific gene expression in mouse cells; processed lamin B1 was required for anchoring gene-poor chromosome 18 at the nuclear periphery and the loss of this anchorage resulted in upregulation of certain genes on this chromosome (Malhas *et al.*, 2007). Alterations in the normal peripheral localization of chromosomes 13 and 18 to a more interior position have been reported in human dermal fibroblasts derived from patients with mutations in lamin A (Meaburn *et al.*, 2007), although such changes have not been observed in lymphoblastoid cells derived from laminopathic patients (Meaburn *et al.*, 2005). A comprehensive survey of genome organization in *Drosophila* has indicated that about 500 genes interact with lamin Dm_0 in embryonic cells (Pickersgill *et al.*, 2006). These genes are transcriptionally inactive and lack histone modifications. Although widely separated in location, developmentally coregulated genes appear to be clustered together, suggesting a dynamic role for the lamina in integrating genomic and epigenetic features.

4.2. Regulation of mitosis

During the prophase–prometaphase transition in animal cells that follow an "open" mitosis, the nuclear envelope, pore complexes, and lamina are disassembled. The reassembly of these structures occurs in a specified sequence toward the end of mitosis. Lamin disassembly and reassembly during nuclear envelope reformation in daughter cells have been studied in detail by various laboratories (Gant and Wilson, 1997). Lamin disassembly is triggered by phosphorylation of essential serine residues flanking the rod domain by the mitotic kinase Cdk1 and may be assisted by

microtubule-induced tearing of the lamina (Beaudouin *et al.*, 2002). Phosphorylation of other components of the nuclear envelope also helps in their dispersal. Lamina reassembly has been studied extensively using both *in vitro* and *in vivo* assays. However, the precise role of lamins in nuclear envelope reformation has been difficult to define because of reported variations in the temporal sequence of events, which may be attributed to cell type-specific differences in the reassembly process. Most data are consistent with the view that assembly of a typical lamina is not required for nuclear membrane and pore assembly, although a small amount of lamins may be necessary for successful envelope formation. In live cell studies, GFP–lamin B1 has been shown to assemble on the periphery of chromosomes during early telophase (Moir *et al.*, 2000a), or after the envelope has reformed in late telophase in certain cell types (Daigle *et al.*, 2001). Lamin A is gradually incorporated into the lamina after envelope formation, during cytokinesis and the early G_1 phase (Broers *et al.*, 1999; Moir *et al.*, 2000a). These differences between the assembly paths of A-type and B-type lamins imply that the two types of lamins might form separate networks. This hypothesis is supported by a study of polymer formation in live cells using fluorescence resonance energy transfer (FRET), which shows that wild-type lamins A and B1 polymerize in distinct homopolymers that subsequently interact in the lamina (Delbarre *et al.*, 2006). An important finding is that lamin B is essential for the formation of a matrix-like network required for the assembly of the mitotic spindle, and a fraction of total lamin B is a functional component of this matrix (Tsai *et al.*, 2006). Interestingly, lamins have been reported to bind to nuclear titin, a protein essential for mitotic chromosome condensation (Zastrow *et al.*, 2006).

4.3. DNA replication

Lamins have been implicated in the organization of DNA replication in several studies. For instance, DNA replication is inhibited in lamin-depleted nuclei (Meier *et al.*, 1991). When N-terminal deletion mutants of human lamin A (Moir *et al.*, 2000b; Spann *et al.*, 1997) or *Xenopus* lamin B1 (Ellis *et al.*, 1997) are added to assembled nuclei, they disrupt lamin organization and inhibit DNA synthesis. Thus a normal lamina is essential for DNA replication in assembled nuclei and cells, although it is possible to replicate DNA *in vitro* with purified factors (Walter *et al.*, 1998). A direct role for lamins in the spatial organization of DNA replication is supported by the colocalization of lamin B with replication foci and replication factors such as proliferating cell nuclear antigen (PCNA) in NIH 3T3 cells during S phase (Moir *et al.*, 1994) and colocalization of lamin A with replication factors at specific stages of S phase in primary mammalian cells (Kennedy *et al.*, 2000).

4.4. Transcription and gene regulation

A possible involvement of lamins in gene regulation was suggested by earlier studies on the developmental and tissue-specific expression of A-type lamins in *Xenopus*, mouse, and *Drosophila* (Benavente *et al.*, 1985; Riemer *et al.*, 1995; Röber *et al.*, 1989; Stick and Hausen, 1985). A-type lamins have been shown to associate with specific transcription factors such as pRb (Mancini *et al.*, 1994; Ozaki *et al.*, 1994); SREBP1 (Lloyd *et al.*, 2002); a Kruppel-like protein, MOK2 (Dreuillet *et al.*, 2002); and, more recently, c-Fos (Ivorra *et al.*, 2006). There is increasing evidence that localization of specific genes at the nuclear periphery may lead to transcriptional silencing. In *Drosophila*, insertion of the *gypsy* insulator into a gene sequence causes its translocation to the nuclear envelope (Gerasimova and Corces, 1998). Association of the POU domain repressor protein Oct-1 with lamin B1 has been correlated with its repressor activity (Imai *et al.*, 1997). Lamin B together with LAP2β forms functional complexes with the transcription factors GCL and DP to repress E2F (de la Luna *et al.*, 1999; Nili *et al.*, 2001).

Compelling evidence of a role for lamins in organization of transcription has been provided by the following studies. Goldman and colleagues have shown that expression of an N-terminal deletion mutant of lamin A, ΔNLA, leads to disruption of the lamina and inhibition of RNA polymerase II (PolII) transcription, without affecting PolI or PolIII transcription (Spann *et al.*, 2002). Parnaik and coworkers have observed that nucleoplasmic lamins termed lamin speckles are relocalized into enlarged domains on treatment with transcriptional inhibitors, and that disruption of these domains in various mammalian cells disorganizes splicing factor compartments and inhibits RNA PolII transcription (Kumaran *et al.*, 2002). Colocalization of lamin speckles with splicing factor compartments has been demonstrated with antibodies that specifically recognize A-type lamins (Jagatheesan *et al.*, 1999; B. Muralikrishna and V. K. Parnaik, unpublished data). Furthermore, RNA interference (RNAi) with lamin A/C disrupts both the peripheral lamina and lamin speckles and also disorganizes splicing factor compartments, suggesting a role for lamin speckles in the maintenance of splicing factor compartments (B.V. Gurudatta and V. K. Parnaik, unpublished data). Splicing factor compartments, also termed interchromatin granule clusters, are dynamic domains involved in the storage and recruitment of splicing factors (Spector, 2003). Previous studies have indicated the presence of lamins in purified preparations of interchromatin granule clusters (Mintz *et al.*, 1999).

There is increasing evidence of a role for other structural proteins such as actin and myosin in transcription. Nuclear actin is involved in transcription and chromatin remodeling (Pederson and Aebi, 2005). Myosins I and VI have been proposed to be required for RNA PolI and PolII transcription,

respectively (Hofmann and de Lanerolle, 2006). Interestingly, actin can bind directly to the C terminus of lamin A (Sasseville and Langelier, 1998).

4.5. Nuclear–cytoskeletal interactions

Processes such as nuclear migration and positioning depend on the microtubule network as well as the actin cytoskeleton. Nuclear migration is a crucial process that occurs during the growth and development of many species. Mutational studies in *C. elegans* have identified a number of SUN domain proteins such as UNC-84, UNC-83, and matefin/SUN-1 (Malone *et al.*, 1999; Malone *et al.*, 2003; Starr *et al.*, 2001) that are involved in nuclear positioning and migration. UNC-84 and matefin/SUN-1 are inner nuclear membrane proteins and the localization of UNC-84 depends on Ce-lamin (Lee *et al.*, 2002). UNC-83, which bears the KASH domain also found in nesprins, is localized at the outer nuclear membrane and physically interacts with UNC-84 (McGee *et al.*, 2006). The giant actin-binding protein ANC-1, which is the *C. elegans* homolog of nesprin-1, is anchored to the outer nuclear membrane by UNC-84 (Starr and Han, 2002). Thus Ce-lamin, UNC-84, and ANC-1 form a complex that can interact with the actin cytoskeleton. In *Drosophila*, expression of a mutant form of lamin Dm_0 leads to a loss in association with klarsicht and microtubules, and results in defective nuclear migration in cells of the eye disc (Patterson *et al.*, 2004). One study has implicated lamin B1 in the anchorage of the nucleus to the cytoskeleton in mammalian embryonic fibroblasts (Ji *et al.*, 2007).

The microtubule-binding protein ZYG-12 is tethered to the outer nuclear membrane via matefin/SUN-1 and also interacts with the centrosome (Malone *et al.*, 2003); this complex is necessary for centrosome migration during mitosis. A mutation in the matefin/SUN-1 gene has been reported to disrupt the reorganization of chromatin during meiosis (Penkner *et al.*, 2007). *C. elegans* UNC-83 and UNC-84 are correctly localized in transfected mammalian cells, suggesting that their targeting mechanisms are conserved (McGee *et al.*, 2006). Studies have described a requirement of the SUN proteins for tethering telomeres to the nuclear envelope during meiosis, which is necessary for homologous chromosome pairing during mammalian gametogenesis (Ding *et al.*, 2007; Schmitt *et al.*, 2007).

4.6. Apoptotic events involving lamins

During the later stages of apoptosis, lamins are cleaved by caspases. This helps the lamina to disassemble and facilitates chromatin condensation (Cohen *et al.*, 2001). The caspase required for cleavage of lamin A has been identified as caspase-6 and the cleavage site is located at amino acid residues 227–230 in the nonhelical linker region L12 in the rod domain. Both lamina assembly and interactions with chromatin are likely to be

disrupted by cleavage of the molecule in this region (Rao *et al.*, 1996; Takahashi *et al.*, 1996). In adenovirus-infected cells, the adenoviral early protein E1B-19K localizes to the ER and nuclear membranes and blocks apoptosis in a lamin-dependent process (Rao *et al.*, 1997). One study suggests that matefin/SUN-1 might provide an important link between the cytoplasmic and nuclear processes of apoptosis during development in *C. elegans* (Tzur *et al.*, 2006). CED-9, the Bcl-2 homolog in *C. elegans*, binds to the Apaf-1 homolog CED-4. This binding interaction is disrupted on initiation of apoptosis and the release of CED-4 results in caspase activation. CED-4 translocates from mitochondria to the nuclear envelope, where it binds specifically to the inner nuclear membrane protein matefin/SUN-1. A role for matefin/SUN-1 in apoptosis is supported by the reduction in the number of apoptotic cells in *C. elegans* embryos after downregulation of matefin/SUN-1 by RNAi.

5. Cellular Signaling Pathways Involving Lamins

A long-standing question concerns how mutations in lamin A, which is expressed in nearly all differentiated tissue types, cause several diseases, most of which are tissue specific. Two prevalent hypotheses that have been proposed to explain the clinical observations are as follows. The ''mechanical stress'' hypothesis states that abnormalities in nuclear structure, resulting from mutations in lamin A, weaken the nuclear lamina–envelope network and thus lead to increased susceptibility to cellular damage by physical stress (Sullivan *et al.*, 1999). The ''gene expression'' hypothesis proposes that nuclear lamin plays a role in tissue-specific gene expression, which can be altered by mutations in lamin A (Wilson, 2000). A number of studies have been carried out with cells from patients and mouse models, as well as transfected cells expressing lamin mutants, to address this problem and these studies have given new insights into the role of lamin A/C in tissue-specific signaling pathways, as is evident from the following discussion.

5.1. Muscle differentiation

There is considerable interest in understanding the role played by A-type lamins in muscle development and the effects of mutations on this process as the majority of mutations in *LMNA* affect muscle tissue. Most studies have focused on the following possibilities. First, it is possible that muscle cells that are constantly under high mechanical stress are unable to survive because of loss of nuclear integrity, leading to loss of cells and a dystrophic phenotype. The second possibility is that lamin A/C plays a specific role in

muscle differentiation and mutations in lamin A/C may affect the muscle differentiation program, leading to aberrant or incomplete differentiation due to misregulation of muscle-specific gene expression, which eventually leads to a dystrophic syndrome. Current evidence suggests that both of these mechanisms may be operative.

Important insights into cellular defects associated with lamin A deficiency, in particular those leading to muscular dystrophy and cardiomyopathy, have been obtained from the *Lmna*$^{-/-}$ mouse model. Fibroblasts from *Lmna*$^{-/-}$ mice have aberrant nuclear morphology, show herniations of the envelope, and mislocalize emerin. In response to mechanical strain, these fibroblasts exhibit increased nuclear deformations and defective mechanotransduction, together with reduced expression of genes activated by NF-κB (Lammerding *et al.*, 2004). *Lmna*$^{-/-}$ mice also develop DCM, and cardiomyocytes from these mice show abnormal nuclear architecture with relocalization of heterochromatin to the nuclear interior and, interestingly, changes in localization of the cytoplasmic filament protein desmin, thus leading to contractile dysfunction (Nikolova *et al.*, 2004). These two studies suggest that absence of lamin A may cause striated muscle disease by impaired nuclear mechanics and secondary changes in gene expression, thus supporting the mechanical stress hypothesis for lamin pathogenesis. A further report on the loss of mechanical stiffness in *Lmna*$^{-/-}$ fibroblasts is consistent with this hypothesis, and also suggests that a loss of physical interactions between lamins and the cytoskeleton may lead to general cellular weakness, making these cells more vulnerable to mechanical stress (Broers *et al.*, 2004).

C2C12 myoblasts stably expressing a common EDMD-causing lamin A mutation, R453W, are deficient in expression of myogenic markers such as myogenin, do not exit the cell cycle properly, and are eventually targeted for apoptosis (Favreau *et al.*, 2004). Further, these cells show persistence of the hyperphosphorylated form of pRb, although pRb is hypophosphorylated during normal myogenesis, and also express the muscle regulator Myf5 at high levels, unlike normal cells. On the other hand, cells expressing the R482W FPLD mutation are able to differentiate normally. Subsequent studies have shown that the EDMD mutation W520S also inhibits myogenin expression and blocks myoblast differentiation (Markiewicz *et al.*, 2005). Parnaik and coworkers have shown that differentiation is strongly impaired in myoblasts expressing the EDMD mutant G232E, Q294P, or R386K and dividing myoblasts expressing these EDMD mutants are deficient in MyoD expression (Parnaik and Manju, 2006).

Primary myoblasts isolated from lamin A/C knockout mice have delayed differentiation kinetics and impaired differentiation (Frock *et al.*, 2006). Certain markers of muscle differentiation such as MyoD and pRb were observed to be downregulated at the protein level whereas the muscle regulator Myf5 was upregulated severalfold. Myf5 has been earlier shown to

be upregulated in mice lacking MyoD (Rudnicki *et al.*, 1992). Interestingly, levels of the cytoskeletal protein desmin were also reduced in about 70% of cells, as observed earlier in *Lmna*$^{-/-}$ cardiomyocytes (Nikolova *et al.*, 2004). Both MyoD and desmin transcript levels were reduced in proliferating *Lmna*$^{-/-}$ myoblasts but pRb transcript levels were normal, suggesting that pRb protein stability was reduced in these cells, perhaps due to the absence of lamin A/C as a binding partner for pRb (Johnson *et al.*, 2004). RNAi-mediated silencing of lamin A/C expression in myoblasts also caused impaired differentiation, and reduction in MyoD and desmin levels, whereas ectopic expression of MyoD or desmin in *Lmna*$^{-/-}$ myoblasts resulted in increased differentiation potential (Frock *et al.*, 2006). It is thus evident that MyoD levels are sensitive to the presence of a normal lamin A network as both depletion of lamin A in *Lmna*$^{-/-}$ myoblasts and over-expression of lamin A constructs, in particular the EDMD mutants, can lead to lowering of MyoD and impairment of muscle differentiation. On the other hand, although *Lmna*$^{-/-}$ myoblasts continue to proliferate on serum deprivation, this is in contrast to the loss in survival of myoblasts expressing EDMD lamin mutants in serum-depleted medium (Favreau *et al.*, 2004; Parnaik and Manju, 2006). A transcriptional profiling of EDMD muscle biopsies suggests a failure of interactions between envelope proteins and pRb and MyoD during exit from the cell cycle (Bakay *et al.*, 2006). Misregulation of pRb/MyoD pathways and delay in muscle regeneration have also been observed in mice that are null for emerin (Melcon *et al.*, 2006). In a study with EDMD cells and cells transfected with lamin mutants, the localization of desmin was observed to be normal (Piercy *et al.*, 2007). Parnaik and colleagues have shown that internal lamins are reorganized into a diffuse, insoluble network before cell cycle arrest during the differentiation of C2C12 myoblasts in culture (Muralikrishna *et al.*, 2001). This diffuse network is also observed in quiescent, satellite cells and transdifferentiated cells but not in nonmuscle cell types. Lamin speckles are induced to rearrange on expression of cyclin D3 in myoblasts and this process also requires pRb; both pRb and cyclin D3 are bound to the insoluble lamin matrix (Mariappan and Parnaik, 2005; Mariappan *et al.*, 2007). Lamin reorganization may be required to maintain the nondividing state of the differentiated myocyte or satellite cell by sequestration of pRb complexes containing cyclin D3.

With the discovery of nuclear membrane-associated proteins such as nesprins and SUNs that have been proposed to bridge the lamina with the cytoskeleton, it can be speculated that muscle-specific gene expression might be influenced by cross-talk between cytoplasmic actin filaments and the nucleoskeleton. In addition, association of the major muscle protein dystrophin with actin filaments as well as plasma membrane protein complexes can provide a continuous link to events at the sarcolemma (see Fig. 4.5). In this context, one study has shown that a mutation in

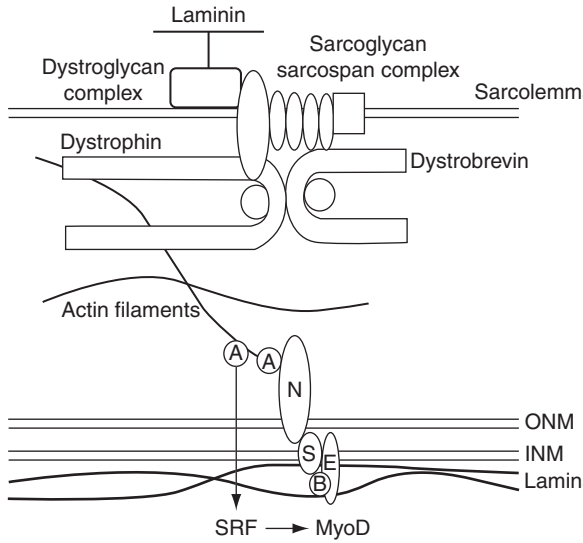

Figure 4.5 Scheme for linkage of proteins at the sarcolemma, cytoskeleton, and nucleoskeleton in muscle cells. This model illustrates known interactions between the muscle proteins dystrophin, dystrobrevin, and sarcolemmal glycoprotein complexes as well as actin filaments (Ervasti, 2007), and proposed links to the nuclear lamina through nesprins (N), SUNs (S), emerin (E), and BAF (B) (see text for references). Networking between nucleoskeletal and cytoskeletal proteins might also hypothetically link the nuclear lamina and muscle-specific gene expression through known effects of actin-binding proteins (A) on activity of serum response factor (SRF) in the regulation of MyoD levels (Pomiès *et al.*, 2007).

δ-sarcoglycan that causes dilated cardiomyopathy leads to mislocalization of lamin A/C and emerin to the nucleoplasm of cardiomyocytes (Heydemann *et al.*, 2007).

5.2. Adipocyte differentiation

LMNA mutations causing FPLD are clustered within exons 8 and 11, which encode the C-terminal domain. Yeast two-hybrid and *in vitro* binding analyses have shown that this region binds specifically to SREBP1 (Lloyd *et al.*, 2002). SREBPs are produced as membrane-bound precursors that reside in the ER and on reduction in cellular cholesterol SREBP1 is proteolytically cleaved and imported into the nucleus, where it directly activates genes involved in cholesterol biosynthesis. SREBP1 has been shown to be sequestered by prelamin A at the nuclear periphery in fibroblasts from lipodystrophy patients, which results in lower pools of active SREBP1 and inhibition of preadipocyte differentiation (Capanni *et al.*, 2005). It has also been suggested that lamin A may normally act as an

inhibitor of adipocyte differentiation, as lamin A-deficient fibroblasts accumulate more intracellular lipid and show elevated triglyceride synthesis compared with wild-type fibroblasts, and overexpression of both wild-type and mutant lamin A inhibits adipocyte differentiation (Boguslavsky *et al.*, 2006). Boguslavsky *et al.* (2006) propose that mutations causing FPLD might be ''gain-of-function'' mutations that result in higher binding affinity for a proadipogenic factor, thereby sequestering it at the nuclear periphery and inhibiting expression of genes involved in adipogenesis. Interestingly, misexpression of the lamin A-binding protein LAP2α alters cell cycle progression and pRb–E2F signaling, and also inhibits adipocyte differentiation (Dorner *et al.*, 2006).

5.3. DNA repair pathways

The initial response to DNA damage by external agents such as ionizing and ultraviolet (UV) irradiation and genotoxic agents, or by internal sources such as reactive oxygen species, involves the chromatin-dependent activation of complex checkpoint signaling pathways in order to delay the cell cycle and repair the defects. The major kinases that serve as damage sensors and regulate cell cycle checkpoints and DNA repair by phosphorylation of key substrates are ataxia-telangiectasia-mutated (ATM) and ATM-and-Rad3-related (ATR) kinases, which belong to the phosphoinositide 3-kinase-related family of serine/threonine protein kinases (Bartek *et al.*, 2004; Shiloh, 2003). ATM and ATR activate cell cycle checkpoints and phosphorylate p53 as well as a number of other downstream targets such as the histone variant H2AX. Mutations in several components of these DNA repair pathways cause premature aging syndromes in humans and cellular senescence in cell culture models. A common premature aging disorder, Werner's syndrome, is caused by loss of a RecQ family DNA helicase, WRN, which functions in several DNA repair pathways. As discussed earlier in this review, mutations in the lamin A gene are associated with atypical Werner's syndrome (Chen *et al.*, 2003) and HGPS (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003). Deletion of the prelamin A processing enzyme Zmpste24 in mice also leads to a progeria-like syndrome (Bergo *et al.*, 2002; Pendás *et al.*, 2002).

Studies with *Zmpste24*-null fibroblasts and HGPS fibroblasts have demonstrated a link between defects in lamin A and the DNA damage response. *Zmpste24*-null fibroblasts show genomic instability, higher sensitivity to DNA-damaging agents, and impairment in recruitment of repair proteins such as p53-binding protein 1 (53BP1) and Rad51 to sites of DNA lesions (Liu *et al.*, 2005). Chromosomal aneuploidy has been observed previously in HGPS cells (Mukherjee and Costello, 1998). An analysis of transcriptional changes in *Zmpste24*-deficient cells has revealed that several targets of the p53 signaling pathway are upregulated, implying that a

checkpoint response is activated by abnormal nuclear architecture (Varela *et al.*, 2005). HGPS fibroblasts also display changes in gene transcription profiles that suggest developmental defects in mesodermal and mesenchymal cell lineages (Csoka *et al.*, 2004b). Parnaik and coworkers have observed that certain lamin mutants impair the formation of phosphorylated H2AX at DNA repair foci and hinder the recruitment of 53BP1 to repair sites after short-term DNA damage. These mutants disrupt emerin localization and, importantly, also mislocalize ATR kinase in untreated cells (Manju *et al.*, 2006). These results suggest that lamin A/C might be required to anchor ATR kinase in the nucleus through interactions with chromatin.

5.4. Transforming growth factor-β-mediated signaling pathways

The transforming growth factor-β (TGF-β) superfamily of cytokines controls signaling pathways involved in cell proliferation and differentiation in many cell types (Massagué *et al.*, 2000). Cytokine-mediated signaling involves binding to cell surface receptors, phosphorylation of Smad proteins, and translocation of Smad complexes into the nucleus, where they regulate a large number of target genes. Studies from several laboratories have reported the involvement of the inner nuclear membrane protein MAN1 (LEMD3) as well as lamin A/C in these pathways. In two separate studies, *Xenopus* MAN1 has been shown to bind to Smad1 and block signaling by bone morphogenetic protein (BMP), which belongs to a subfamily of TGF-β cytokines (Osada *et al.*, 2003; Raju *et al.*, 2003). MAN1 can also bind to Smads 1, 2, 3, and 5 in mammalian cells (Lin *et al.*, 2005; Pan *et al.*, 2005), and these interactions lead to inhibition of signaling by TGF-β, activin, and BMP. MAN1 is able to block phosphorylation and nuclear translocation of Smads (Pan *et al.*, 2005; Raju *et al.*, 2003). Hellemans *et al.* (2004) have demonstrated that fibroblasts from human patients with mutations in MAN1 express higher levels of genes regulated by BMP and TGF-β. Van Berlo *et al.* (2005) have observed that A-type lamins interact with protein phosphatase 2A and may thus modulate TGF-β_1 signaling. Hence mutations in lamin A might affect TGF-β signaling pathways directly or indirectly through MAN1.

5.5. Cellular proliferation

It is becoming increasingly evident that nuclear structural proteins play an important role in the control of cell proliferation. pRb is a critical cell cycle regulator, which is involved in controlling cell proliferation as well as terminal differentiation (Korenjak and Brehm, 2005). During the early G_1 phase, pRb is hypophosphorylated and tethered to the nuclear matrix (Mancini *et al.*, 1994), and binds directly to lamin A/C and LAP2α

(Ozaki *et al.*, 1994; Markiewicz *et al.*, 2002). pRb is rapidly degraded in $Lmna^{-/-}$ fibroblasts, suggesting that A-type lamins are required for pRb stability. Also, cell cycle parameters of $Lmna^{-/-}$ fibroblasts such as rapid proliferation, higher proportion of cells in S phase, and reduced contact inhibition are similar to those of $Rb^{-/-}$ cells (Johnson *et al.*, 2004). A-type lamins are necessary for proper cell cycle exit mediated by the Cdk inhibitor $p16^{ink\text{-}4a}$, which also requires pRb for this process (Nitta *et al.*, 2006). Because both lamin A/C and LAP2α can bind to pRb, the involvement of LAP2α in cell cycle events mediated by pRb has been investigated. Overexpression of LAP2α in 3T3 fibroblasts leads to reduction in cell proliferation and delays cell cycle entry of G_0-arrested cells whereas down-regulation of LAP2α increases cell proliferation and hinders cell cycle arrest (Dorner *et al.*, 2006). Furthermore, LAP2α inhibits the expression of E2F target genes in a pRb-dependent manner. The phosphorylation status and subnuclear localization of pRb has been proposed to depend on lamin A/C as well as LAP2α (Pekovic *et al.*, 2007). In HGPS cells, the Rb-mediated G_1–S phase transition is impaired, probably because of inhibition of phosphorylation of pRb by Cdk4 kinase (Dechat *et al.*, 2007); these cells also display abnormal localization of progerin during mitosis and mitotic defects (Cao *et al.*, 2007; Dechat *et al.*, 2007).

A-type lamins have been shown to negatively regulate the functions of AP-1 transcription factor by inhibition of c-Fos/c-Jun heterodimerization (Ivorra *et al.*, 2006). In mitogen-activated cells, there is substantial accumulation of nucleoplasmic c-Fos that is available for AP-1 heterodimer formation and transcriptional activation. On serum starvation, lamin A/C sequesters residual c-Fos at the nuclear periphery, thereby suppressing its activity.

6. Concluding Remarks

Lamins play a crucial role in maintaining nuclear integrity and are involved in the organization of nuclear functions such as DNA replication, transcription, and repair. The association of lamins with nuclear membrane proteins is likely to provide an interconnecting network between the cytoskeleton and the nucleoskeleton, with interesting possibilities for control of cellular signaling. Studies with laminopathic mutations in both cellular and animal models have given valuable insights into the role of lamins in cellular signaling pathways involved in differentiation, proliferation, and aging. An emerging concept is that lamins are required to sequester key regulatory factors along specific pathways. Although the C-terminal mutations that cause FPLD affect primarily adipocyte differentiation, it is becoming increasingly evident that certain mutations in lamin A/C are able

to affect multiple cellular processes, with highly deleterious mutations such as lamin AΔ50 leading to general cellular toxicity and cell death. There has been intensive focus on this mutation, with newer findings supporting a role in normal aging as well as HGPS. A developing notion is that lamin mutations may lead to deregulation of pRb, which would impair its regulatory roles in cellular proliferation, muscle differentiation, adipocyte differentiation, and cellular senescence. The effects of impaired differentiation on adult tissue repair and regeneration need further analysis.

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CHAPTER FIVE

New Insights into the Mechanisms of Macroautophagy in Mammalian Cells

Eeva-Liisa Eskelinen

Contents

Abstract

Macroautophagy is a self-digesting pathway responsible for the removal of long-lived proteins and organelles by the lysosomal compartment. Parts of the cytoplasm are first segregated in double-membrane-bound autophagosomes, which then undergo a multistep maturation process including fusion with endosomes and lysosomes. The segregated cytoplasm is then degraded by the lysosomal hydrolases. The discovery of ATG genes has greatly enhanced our understanding of the mechanisms of this pathway. Two novel ubiquitin-like protein conjugation systems were shown to function during autophagosome formation. Autophagy has been shown to play a role in a wide variety of

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physiological processes including energy metabolism, organelle turnover, growth regulation, and aging. Impaired autophagy can lead to diseases such as cardiomyopathy and cancer. This review summarizes current knowledge about the formation and maturation of autophagosomes, the role of macroautophagy in various physiological and pathological conditions, and the signaling pathways that regulate this process in mammalian cells.

Key Words: Autophagy, Protein turnover, ATG proteins, Lysosome, LAMP-2, Rab7, Stress response.

1. Introduction

Autophagy is an evolutionarily conserved and strictly regulated lysosomal pathway that degrades cytoplasmic material and organelles. This pathway is activated under stress conditions such as amino acid starvation, unfolded protein response, or viral infection. Depending on the delivery route of the cytoplasmic material to the lysosomal lumen, four different autophagic routes are known: (1) macroautophagy, or simply autophagy, (2) microautophagy, (3) chaperone-mediated autophagy, and (4) crinophagy (Fig. 5.1). In macroautophagy, a portion of cytoplasm to be degraded is first wrapped inside a specialized organelle, the autophagosome, which then fuses with lysosomal vesicles and delivers the engulfed cytoplasm for degradation (Arstila and Trump, 1968) (Fig. 5.2). In microautophagy, the lysosomal membrane itself sequesters a portion of cytoplasm by a process

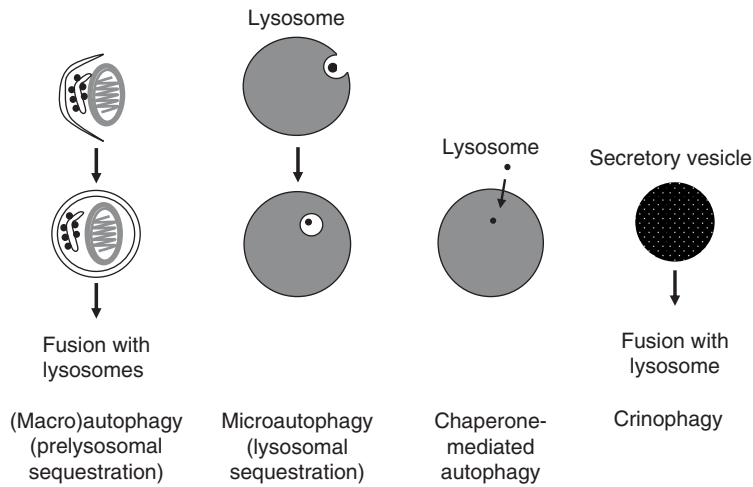

Figure 5.1 Schematic presentation of autophagic segregation pathways.

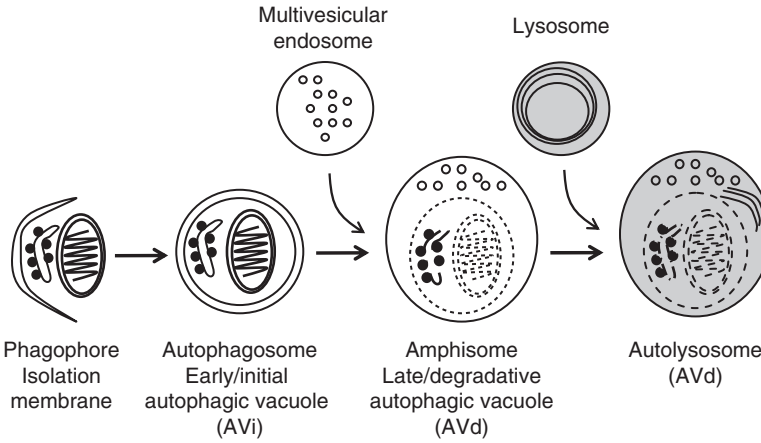

Figure 5.2 Schematic presentation of the macroautophagic pathway. The nomenclature of the different maturation stages is indicated.

that resembles pinching off of phagosomes or pinosomes from the plasma membrane (Ahlberg *et al.*, 1982). Starvation-induced macroautophagic uptake of cytoplasmic material appears to be a nonselective process (Kopitz *et al.*, 1990); organelles are sequestered at the same frequency as they exist in the cytoplasm. In chaperone-mediated autophagy, proteins possessing a specific sequence signal are transported from the cytoplasm, through the lysosomal membrane, to the lysosomal lumen (Cuervo and Dice, 1996). The lysosome-associated membrane protein (LAMP)-2 was proposed to act as a receptor in chaperone-mediated autophagy. A fourth autophagic route, crinophagy, has also been described (Glaumann, 1989). In crinophagy, secretory vesicles directly fuse with lysosomes, which leads to degradation of the granule contents. This review concentrates on the (macro)autophagic pathway in mammalian cells.

After induction by a stress signal such as amino acid starvation, the first step in (macro)autophagy is the formation of an *autophagosome* (Fig. 5.2). A flat membrane cistern elongates and wraps itself around a portion of cytoplasm, forming a double-membrane-bound autophagosome. This membrane cistern has been called the *phagophore*, or *isolation membrane*. Autophagosomes next receive lysosomal constituents, such as lysosomal membrane proteins and proton pumps, from endosomal vesicles via vesicle-mediated transport, and/or by fusion with late endosomes or multivesicular bodies (MVBs). Finally, the limiting membranes of autophagosomes fuse with the limiting membranes of lysosomes (Berg *et al.*, 1998; Dunn, 1990b; Gordon *et al.*, 1992; Lawrence and Brown, 1992; Liou *et al.*, 1997; Punnonen *et al.*, 1993; Tooze *et al.*, 1990) (Fig. 5.2). In this process, the cytoplasm, still engulfed by the inner limiting membrane, is delivered to

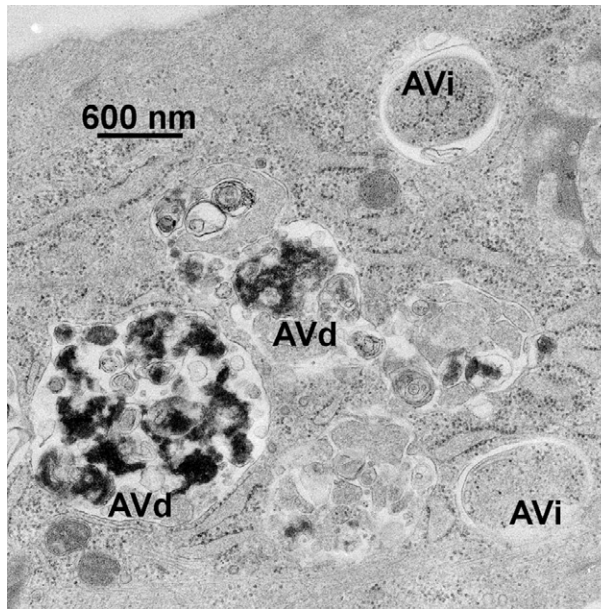

Figure 5.3 Transmission electron microscopy demonstrating the ultrastructure of autophagic vacuoles in a mouse fibroblast. The cells were incubated in serum-and amino acid-free medium for 2 h before fixation for microscopy. Early autophagic vacuoles (AVi) contain morphologically intact cytoplasm. Degradative autophagic vacuoles (AVd) contain partially degraded cytoplasmic material, above all remnants of ribosomes, which are electron dense.

the endo/lysosomal lumen (Figs. 5.2 and 5.3). Both the cytoplasm and the membrane around it are then degraded by lysosomal hydrolases, and the degradation products are transported back to cytoplasm, where they can be reused for metabolism. In yeast cells, autophagosomes directly fuse with the vacuole, the counterpart of the mammalian lysosome, without an endosomal fusion step (Baba *et al.*, 1994).

By definition, autophagosomes, also called *initial autophagic vacuoles* (AVi), do not yet contain lysosomal membrane proteins or enzymes, and are not acidic (Dunn, 1990a). During the maturation process, autophagosomes develop into *late*, or *degradative autophagic vacuoles* (AVd), which are acidic and contain lysosomal membrane proteins and enzymes (Dunn, 1990b) (Fig. 5.3). After fusion with lysosomes, autophagosomes are called *autolysosomes*. Quantitative immunoelectron microscopy has been used to demonstrate the enrichment of lysosomal membrane proteins and enzymes in late autophagic vacuoles/autolysosomes (Eskelinen *et al.*, 2002a; Tanaka *et al.*, 2000).

The lack of integral membrane proteins in autophagosomes (AVi) was first revealed by freeze–fracture electron microscopy. Other cellular membranes such as lysosomal and endoplasmic reticulum membranes contain

numerous integral membrane particles, considered to represent integral membrane protein molecules revealed by the freeze–fracture procedure. However, the surfaces of the membranes limiting autophagosomes are almost completely smooth (Fengsrud *et al.*, 2000; Punnonen *et al.*, 1989; Rez and Meldolesi, 1980).

The origin of the membrane cistern forming new autophagosomes has been the subject of numerous studies, but still this issue is unresolved in mammalian cells. Many older ultrastructural studies suggested that smooth endoplasmic reticulum (ER) cisternae are the source of autophagosome membranes (Dunn, 1994), but evidence against this interpretation has also been published (Yamamoto *et al.*, 1990). Studies in yeast have revealed that autophagosomes originate from a unique compartment called the preautophagosomal structure (PAS) (Kim *et al.*, 2002; Suzuki *et al.*, 2001). Because the autophagic pathway and genes involved in it are well conserved from yeast to mammals, it is possible that a similar or equivalent unique compartment is the source of membrane in mammalian cells.

Interestingly, ER cisternae are often observed to closely surround nascent autophagosome membranes (Fig. 5.4). In yeast, membrane transport out of the ER seems to contribute to autophagosome formation. Coat protein complex II (COPII)-coated vesicles transport material from the ER to the Golgi complex. Yeast mutants defective in the Sec23–Sec24 subcomplex of COPII vesicles are unable to form autophagosomes. However, mutants defective in Sec12 or the Sec23–Sec31 subcomplex (also needed for COPII vesicle formation) have no autophagy defects (Hamasaki *et al.*, 2003; Ishihara *et al.*, 2001). Trs85, a component of the TRAPP complexes, is also required for autophagy in yeast (Meiling-Wesse *et al.*, 2005; Nazarko *et al.*, 2005). The transport protein particle, or TRAPP, is a complex of 10 subunits that is essential for tethering of ER-derived transport vesicles to Golgi membranes. Further, conventional membrane fusion machinery, including *N*-ethylmaleimide-sensitive fusion protein [or *N*-ethylmaleimide-sensitive factor (NSF)], soluble NSF attachment protein (SNAP), and SNAP receptors, are not needed for autophagosome formation in yeast (Ishihara *et al.*, 2001; Suzuki and Ohsumi, 2007). These results suggest that unconventional membrane traffic pathways are used during autophagosome formation.

Starvation-induced macroautophagic uptake of cytoplasmic material has been considered a nonselective process (Kopitz *et al.*, 1990). However, selective autophagic uptake of peroxisomes (pexophagy) has been described in yeast (Bellu and Kiel, 2003; Hutchins *et al.*, 1999). Also in mammalian cells, autophagy seems to be necessary for the removal of excess peroxisomes (Iwata *et al.*, 2006), but this uptake is not as strictly selective for peroxisomes as pexophagy in yeast. A selective sequestration of the endoplasmic reticulum was described during the unfolded protein response in yeast (Bernales *et al.*, 2006). Unfolded protein response triggers autophagy also in mammalian cells (Ogata *et al.*, 2006; Yorimitsu *et al.*, 2006) but this uptake does not seem to be

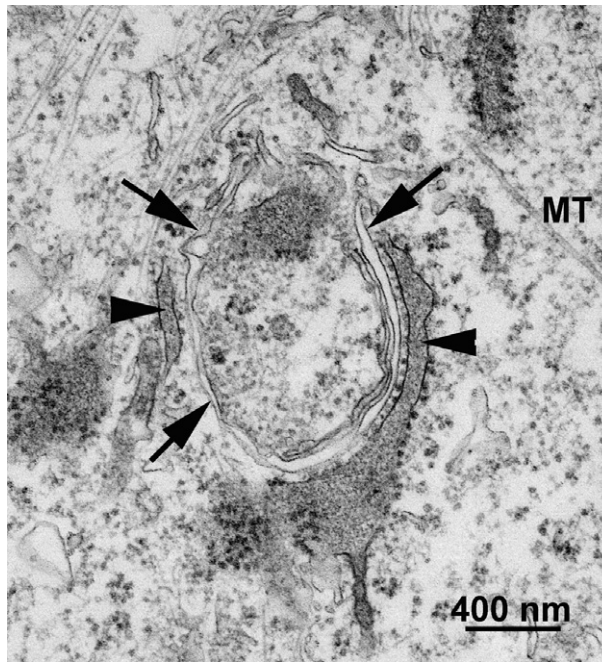

Figure 5.4 Transmission electron microscopy demonstrating the ultrastructure of a putative phagophore in a mouse fibroblast. Cells were incubated in serum- and amino acid-free medium for 2 h before fixation for microscopy. Arrows indicate the membrane of the phagophore. Arrowheads point to a cistern of rough endoplasmic reticulum that tightly surrounds the forming autophagosome.

strictly selective for the ER, although it is possible that the ER is enriched in autophagosome contents. There is also evidence for a selective uptake of mitochondria by macroautophagy in mammalian hepatocytes (Elmore *et al.*, 2001; Kim *et al.*, 2007). This selective uptake has been called mitophagy and was suggested to be important for the removal of dysfunctional mitochondria. The mechanism of this proposed selectivity is currently unknown. It is, however, possible that reactive oxygen species (ROS) might play a role in the recognition of mitochondria for segregation (Scherz-Shouval *et al.*, 2007) (see Section 4.1.2).

2. Autophagy Proteins and Their Known Functions

Yeast genetics has immensely enlarged our knowledge of the molecular mechanisms involved in autophagy (Klionsky, 2004). Today, about 30 autophagy-related yeast genes are known, and numerous reviews have been

written on their functions (Nair and Klionsky, 2005; Suzuki and Ohsumi, 2007; Yorimitsu and Klionsky, 2005). Originally these genes were called APG, AUT, or CVT genes, but a consensus has now been reached, with all autophagy-related genes being described by a common name, ATG (Klionsky *et al.*, 2003). Several mammalian homologs of yeast autophagy genes have been identified (summarized in Table 5.1), and it has been shown that the mechanisms of yeast autophagy are largely conserved in mammals. Two novel protein conjugation systems were shown to be necessary for autophagosome formation, the Atg12–Atg5 conjugation and Atg8–phosphatidylethanolamine conjugation systems (Ohsumi, 2001). The mechanisms of both conjugation systems closely resemble ubiquitin conjugation to proteins, with corresponding conjugation-assisting enzymes to the E1 and E2 enzymes in ubiquitin conjugation (Fig. 5.5).

2.1. Atg12–Atg5

In yeast, autophagosomes seem to arise from a novel perivacuolar compartment, the preautophagosome structure or PAS (Kim *et al.*, 2002; Suzuki *et al.*, 2001). The mammalian autophagy protein Atg5 localizes to small crescent-shaped membrane structures that may be the mammalian PAS, the compartment that delivers membrane for newly forming autophagosomes (Mizushima *et al.*, 2001). In developing autophagosomes, Atg5 localized mainly to the cytoplasmic side of the outer limiting membrane. Atg5 was not present on the membranes of sealed autophagosomes. After synthesis Atg5 is covalently conjugated to Atg12 by a lysine located in the middle of Atg5 (Mizushima *et al.*, 1998). This reaction is similar to ubiquitin conjugation: Atg7 acts as an equivalent to the ubiquitin-activating enzyme E1, and Atg10 acts as an equivalent to the E2 enzyme in this reaction (Mizushima *et al.*, 2003b) (Fig. 5.5). The Atg12–Atg5 conjugates are then linked to a polymer by a third protein, Atg16L (Mizushima *et al.*, 2003a,b). It was proposed that this polymer acts as a membrane coat, which assists membrane curvature during autophagosome formation. Autophagosomes do not form without Atg5 protein, and they also do not form with a mutated Atg5 protein that is not able to form a conjugate with Atg12.

2.2. Atg8/LC3

The ubiquitin-like protein Atg8 is necessary for the formation of normal-sized autophagosomes in yeast (Abeliovich *et al.*, 2000). MAP-1 LC3 is the mammalian homolog of yeast Atg8, and it was the first autophagosome marker protein to be identified (Kabeya *et al.*, 2000). This protein was originally identified as a microtubule-associated protein and named "microtubule-associated protein 1 light chain 3" (Kuznetsov and Gelfand, 1987). LC3 is a small (16- to 18-kDa) protein that is soluble under normal

Table 5.1 Known Mammalian Autophagy Proteins

Mammalian autophagy protein	Yeast protein (old/new name)	Reference(s)	Function of protein
ULK1/Atg1	Apg1/Aut3/ Cvt10/Atg1	Chan *et al.* (2007)	Ser/Thr kinase; signal transduction
Atg3	Apg3/Aut1/ Atg3	Tanida *et al.* (2002b)	Autophagosome formation; mediates LC3 modification and conjugation of Atg5 to Atg12
Atg4/ autophagins	Apg4/Aut2/ Atg4	Hemelaar *et al.* (2003); Marino *et al.* (2003)	Autophagosome formation; assists LC3 modification by cleaving the C terminus to expose glycine
Atg5	Apg5/Atg5	Mizushima *et al.* (2001)	Autophagosome formation; localizes to isolation membranes that are forming new autophagosomes. Forms a complex with Atg12
Beclin 1	Apg6/Vps30/ Atg6	Kihara *et al.* (2001a); Liang *et al.* (1999, 2001); Tassa *et al.* (2003)	Autophagy induction or autophagosome formation; forms a complex with class III PI3-kinase Vps34. A tumor suppressor gene in mammals
Atg7	Apg7/Atg7	Tanida *et al.* (2001, 2002c)	Autophagosome formation; mediates conjugation of Atg5 to Atg12, and LC3 modification

Table 5.1 (*continued*)

Mammalian autophagy protein	Yeast protein (old/new name)	Reference(s)	Function of protein
MAP-LC3	Aut7/Apg8/ Atg8	Kabeya *et al.* (2000)	Autophagosome formation; localizes to the limiting membranes of autophagosomes
Atg9	Apg9/Aut9/ Cvt7	Young *et al.* (2006)	Membrane delivery to forming autophagosomes?
Atg10	Apg10/Atg10	Mizushima *et al.* (2002); Nemoto *et al.* (2003)	Autophagosome formation; mediates conjugation of Atg5 to Atg12 and facilitates LC3 modification
Atg12	Apg12/Atg12	Tanida *et al.* (2002a)	Forms a complex with Atg5
Atg16L	Apg16/Atg16	Mizushima *et al.* (2003a)	Autophagosome formation; links together Atg12–Atg5 complexes to form polymers
WIPI-1	Aut10/Cvt1/ Atg18	Proikas-Cezanne *et al.* (2004)	Autophagosome formation?

conditions, but becomes peripherally membrane associated during autophagy induction (Fig. 5.6A and B). By immunoelectron microscopy, LC3 was shown to associate with the inner and outer limiting membranes of autophagosomes (Kabeya *et al.*, 2000) (Fig. 5.7). During maturation of autophagosomes to autolysosomes, LC3 seems to dissociate from the outer limiting membrane (Jäger *et al.*, 2004; Kabeya *et al.*, 2000), while the LC3 trapped inside autophagosomes is delivered to lysosomes for degradation. In Western blots, two forms of LC3 are seen: LC3-I and LC3-II (Fig. 5.6C and D). After cell homogenization and differential centrifugation, LC3-I is found in the soluble fraction, and LC3-II in the pelletable membrane fraction (Kabeya *et al.*, 2000). The membrane

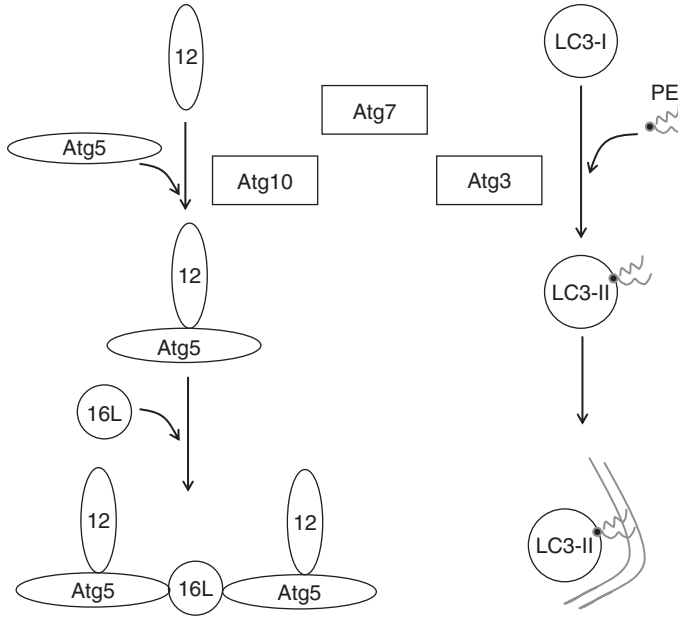

Figure 5.5 Schematic presentation of the ubiquitin-like protein conjugation systems in autophagy. The Atg proteins 7, 10 and 3 (boxed) represent E1- and E2-equivalent enzymes that assist the conjugation. The E1 equivalent Atg7 assists in both conjugation reactions, whereas the E2 equivalent Atg10 is specific for Atg12–Atg5, and Atg3 is specific for LC3–phosphatidylethanolamine (PE) conjugation. The Atg12–Atg5 conjugate is linked to a polymer by Atg16L. After PE conjugation, LC3-II associates with a membrane, which is indicated by the gray lines.

association is mediated by a covalent conjugation to a lipid, phosphatidylethanolamine (Ichimura *et al.*, 2000; Kabeya *et al.*, 2004; Sou *et al.*, 2006). In the sequential chain of events, Atg4 first cleaves the C terminus of LC3 to expose a glycine required for the conjugation. Atg7 next acts as the E1 enzyme, and then Atg3 as the E2 enzyme, during the lipidation of LC3 (Fig. 5.5). At a later stage, Atg4 is also able to remove the attached lipid, releasing soluble LC3-I (Mizushima *et al.*, 2003b). One study showed that lipidated yeast Atg8 mediated tethering and fusion of membranes (Nakatogawa *et al.*, 2007). The authors proposed that membrane fusion may be the authentic function of Atg8 during autophagosome formation.

Both LC3-I and LC3-II are seen in nonstarved mammalian cells, but during autophagy induction the proportion of LC3-II increases (Kabeya *et al.*, 2000), especially if lysosomal degradation is inhibited with drugs (Tanida *et al.*, 2005) (Fig. 5.6D). In addition to LC3, two other Atg8 homologs exist in mammals: GATE16 (Golgi-associated ATPase enhancer of 16 kDa) and GABARAP [γ-aminobutyric acid (GABA) type A receptor-associated protein] (Tanida *et al.*, 2001). Both of these proteins have been

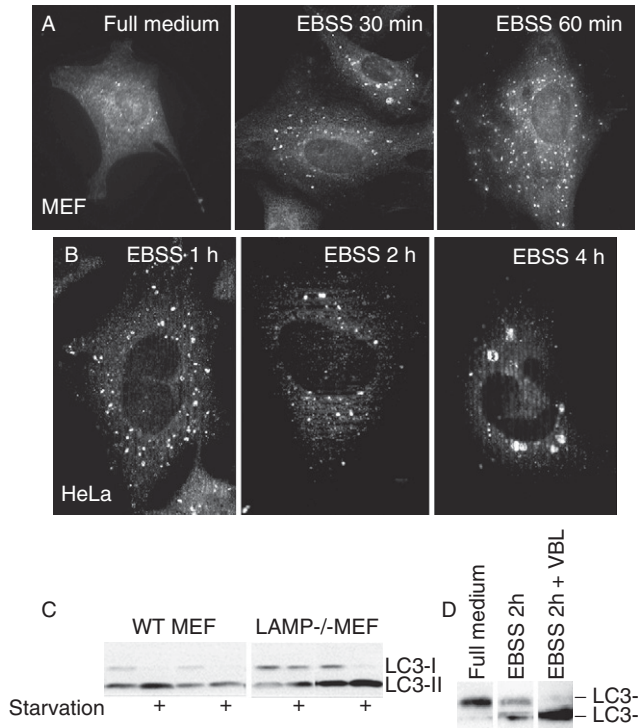

Figure 5.6 Endogenous LC3 as a marker for autophagosome formation. (A and B) Immunofluorescence staining of endogenous LC3 in mouse embryonic fibroblasts (MEFs) (A) and HeLa cells (B). Cells were either fixed without treatment or incubated in serum- and amino acid-free medium [Earle's balanced salt solution (EBSS)] before fixation, as indicated in the panels. Note the increase in the number of LC3 dots until 60 min of incubation (A), as well as the reduction in number, and increase in size, of the dots during longer incubation (B). (C and D) Western blotting of endogenous LC3 in mouse embryonic fibroblasts (C) and HeLa cells (D). In (C), starvation was performed in serum- and amino acid-free medium for 2 h. Wild-type (WT) and LAMP-1/LAMP-2 double-deficient ($LAMP^{-/-}$) cells are shown. In (D), HeLa cells were starved in EBSS alone, or in EBSS containing 50 μ*M* vinblastine (VBL), as indicated. (A) Photos by Päivi Ylä-Anttila; (B and D) photos by Stefanie Jäger; (C) photos by Christine Schmidt. Anti-LC3 was provided by Isei Tanida and Takashi Ueno.

localized to autophagosomes (Kabeya *et al.*, 2004), but it is not known whether they play a role similar to LC3 in autophagosome formation.

2.3. Beclin 1 and Vps34

The tumor suppressor gene Beclin 1 is the mammalian homolog of Atg6. Both mammalian Beclin 1 and yeast Atg6 form a complex with the phosphatidylinositol 3-kinase (PI3-kinase) Vps34. The yeast Vps34 is found in

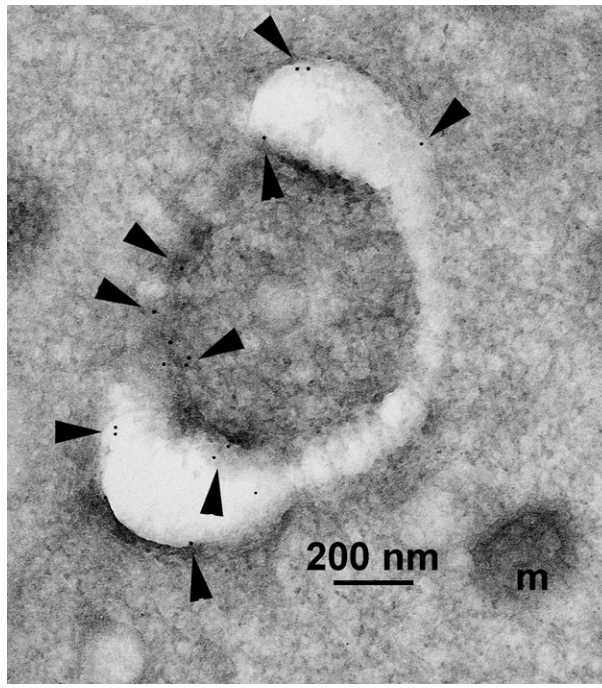

Figure 5.7 Immunogold electron microscopy of endogenous LC3 in a mouse embryonic stem cell. Samples were prepared for labeling by Tokuyasu cryosectioning. Rabbit anti-LC3 was detected with goat anti-rabbit IgG coupled to 10-nm gold particles (arrowheads). Note the presence of LC3 on both the outer and inner limiting membrane of the autophagosome. m, Mitochondrion. Photo by Päivi Ylä-Anttila. Anti-LC3 was provided by Isei Tanida and Takashi Ueno.

two different complexes. The autophagy-specific complex I contains Vps34, Vps15, Atg6, and Atg14. The second complex, containing Vps34, Vps15, Atg6, and Vps38, functions in vacuolar sorting (Kihara *et al.*, 2001b). Beclin 1 forms a complex with the class III PI3-kinase Vps34, also called PI3KC3 (Kihara *et al.*, 2001a; Tassa *et al.*, 2003). In mammals, the serine/threonine kinase Vps15 is represented by p150 (Panaretou *et al.*, 1997). This enzyme is myristoylated and thought to mediate the membrane association of the Beclin 1–Vps34 complex. In addition, UVRAG (UV irradiation resistance-associated gene) was reported to associate with the Beclin 1–Vps34 complex and to enhance autophagy (Liang *et al.*, 2006). The mammalian counterpart of the fourth yeast Vps34 complex I component (Atg14) is currently being sought and it is possible that it may turn out to be UVRAG. In mammalian cells, like in yeast, both Beclin 1 and Vps34 are needed for autophagosome formation (Eskelinen *et al.*, 2002b; Liang *et al.*, 1999; Petiot *et al.*, 2000). However, the precise roles of this complex in autophagosome formation are not known. It is possible that the role of

Vps34 is in the signal transduction events that initiate autophagy, or very early during autophagosome formation. This suggestion is in line with the results showing that in yeast, Atg6 and Atg14 functions are needed before the functions of the Atg12–Atg5 complex (Suzuki *et al.*, 2007).

2.4. Atg9

Atg9, unlike most of the Atg proteins, is a transmembrane protein. In starving yeast cells, Atg9 localizes to the PAS but not to autophagosomes (Kim *et al.*, 2002; Noda *et al.*, 2000). Together with two other Atg proteins, yeast Atg9 has been proposed to recycle between the PAS and a peripheral, non-PAS compartment (Legakis *et al.*, 2007). Because Atg9 is a transmembrane protein, it has been suggested to help in defining the membrane source of the PAS and autophagosomes. Mammalian Atg9 localizes to the trans-Golgi network and late endosomes. Induction of autophagy caused redistribution of Atg9 from the Golgi region to endosomal membranes that were also positive for LC3 (Young *et al.*, 2006). This redistribution was dependent on phosphatidylinositol 3-kinase activity and ULK1, the mammalian homolog of Atg1.

2.5. Atg15

Yeast Atg15 is needed for the intravacuolar lysis of autophagocytosed cytoplasm (Epple *et al.*, 2001; Teter *et al.*, 2001). Atg15 is a putative lipase, although the substrate specificity is unknown. In the absence of Atg15, the inner limiting membranes of autophagosomes, which are delivered into the vacuole with the segregated cytoplasm, stay intact. Atg15 is delivered to the vacuole via multivesicular endosomes, and Atg15 unable to leave the endoplasmic reticulum does not support lysis of autophagocytosed material (Epple *et al.*, 2003). This suggests that the fusion of multivesicular endosomes with the vacuole is essential to initiate the degradation of segregated cytoplasm. The mammalian homolog of Atg15 is presently unknown, but autophagosomes are frequently observed to fuse with multivesicular endosomes (Eskelinen, 2005) (Fig. 5.8). Further, one study showed that autophagosome maturation is impaired if Hrs, a protein needed for the biogenesis of multivesicular endosomes, is downregulated by RNA interference (Tamai *et al.*, 2007) (see Section 3.2.4).

3. Maturation of Autophagosomes into Autolysosomes

3.1. Multistep maturation process

Most of the currently known yeast and mammalian autophagy proteins function during the early steps of autophagy induction or autophagosome formation. On the other hand, less is known about the proteins that regulate

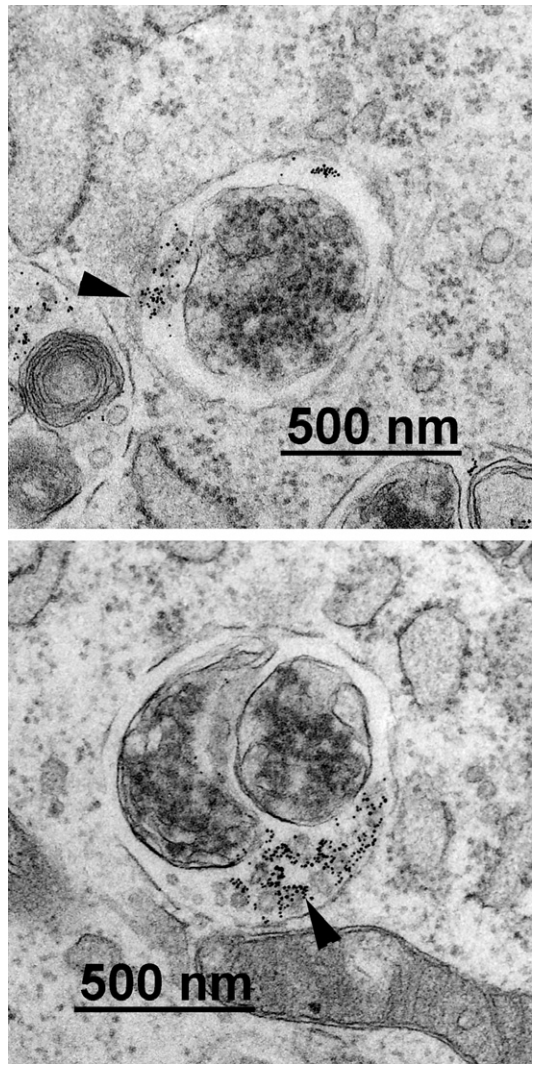

Figure 5.8 Ultrastructure of amphisomes in mouse fibroblasts. To trace endosomal vesicles, cells were incubated in the presence of bovine serum albumin-coated 6-nm gold particles in serum-free medium for 2 h before fixation. Arrowheads indicate endocytosed gold tracer inside autophagic vacuoles, demonstrating that these have fused with endosomes. Note also the small vesicles among the gold particles, indicating the fusion partner was a multivesicular endosome.

the fusion events during maturation of autophagosomes into degradative autolysosomes. The following sections summarize the older knowledge on the maturation of autophagic vacuoles, and describe findings on the proteins regulating this process.

The limiting membranes of autophagosomes contain only trace amounts of lysosomal membrane proteins. This has been shown both by immunogold labeling (Eskelinen *et al.*, 2002b; Liou *et al.*, 1997; Tanaka *et al.*, 2000; Tooze *et al.*, 1990) and by subcellular fractionation (Berg *et al.*, 1998). These proteins are, however, present in the limiting membranes of late autophagic vacuoles (Dunn, 1990b; Eskelinen *et al.*, 2002b; Liou *et al.*, 1997; Tanaka *et al.*, 2000; Tooze *et al.*, 1990), indicating that they are delivered during the maturation process. The same applies to membrane cholesterol. Phagophores have no detectable cholesterol, but the cholesterol content increases during the maturation to early and late autophagic vacuoles (Eskelinen, 2005; Punnonen *et al.*, 1989). Like the lysosomal membrane proteins (Eskelinen *et al.*, 2002a; Jäger *et al.*, 2004), cholesterol was detected mainly in the outer limiting membrane of late autophagic vacuoles.

Autophagosomes or autophagic vacuoles have been reported to fuse with early (Liou *et al.*, 1997; Tooze *et al.*, 1990) and late endosomes (Berg *et al.*, 1998; Lucocq and Walker, 1997; Punnonen *et al.*, 1993) as well as lysosomes (Dunn, 1990b; Gordon *et al.*, 1992; Lawrence and Brown, 1992). These results indicate that the maturation of autophagosomes in mammalian cells is a multistep process including several fusion events with vesicles originating from the endo/lysosomal compartment. A schematic model on the sequential fusion events is presented in Fig. 5.2. According to this model autophagosomes fuse with multivesicular endosomes, forming *amphisomes* (Fig. 5.8). Finally amphisomes fuse with lysosomes. Lysosomal membrane proteins and enzymes are present in both late endosomes and lysosomes (Griffiths *et al.*, 1988), indicating that these proteins can be delivered to autophagic vacuoles during fusion with either of them. Multivesicular endosomes (also called multivesicular bodies) can be either endosomal carrier vesicles or late endosomes (Kobayashi *et al.*, 2002). The model in Fig. 5.2 predicts that autophagosomes (AVi) do not contain lysosomal membrane proteins or enzymes, whereas both amphisomes and autolysosomes (AVd) do, which is in agreement with experimental data (Liou *et al.*, 1997; Tanaka *et al.*, 2000; Tooze *et al.*, 1990).

Originally autophagosomes have the same pH as the surrounding cytoplasm, but during the maturation autophagic vacuoles become acidic (Dunn, 1990b; Punnonen *et al.*, 1992). In mouse hepatocytes, the pH values of AVi and AVd were estimated to be 6.4 and 5.7, respectively (Tanaka *et al.*, 2000). It has been suggested that acidification begins before the delivery of lysosomal enzymes, via fusion with vesicles containing lysosomal membrane proteins and proton pumps, but no lysosomal enzymes (Dunn, 1990b). It is also possible that the proton pumps are delivered to autophagosomes by fusion with endosomes or other autophagic vacuoles.

In the yeast *Saccharomyces cerevisiae*, autophagosomes seem to undergo direct fusion with the vacuole (yeast lysosome) without any preceding fusion with endosomal vesicles (Noda and Ohsumi, 2004). The inhibition

of autophagosome–vacuole fusion can thus be detected as accumulation of autophagosomes in the cytoplasm. Amphisomes are not detectable in yeast. Autophagosomes were unable to fuse with the vacuole in mutants lacking Ypt7 (the yeast homolog of Rab7) (Kirisako *et al.*, 1999); Vam3 (a syntaxin homolog) (Darsow *et al.*, 1997); Sec18 (yeast homolog of *N*-ethylmaleimide-sensitive factor [NSF]); Vti1, an SNARE [SNAP (soluble NSF attachment protein) receptor] protein (Ishihara *et al.*, 2001); Mon1/Aut12 (Meiling-Wesse *et al.*, 2002b); Ccz1/Aut11/Cvt16 (Meiling-Wesse *et al.*, 2002a); or Aut10/Atg18 (Barth *et al.*, 2001). Excluding Vti1 and Ypt7 (Rab7) (see Sections 3.2.5 and 3.2.6, respectively), the roles of these proteins in mammalian autophagy, if any, are unknown.

3.2. Factors required for autophagosome maturation in mammalian cells

3.2.1. Inhibitory drugs

Several drugs have been discovered to inhibit autophagosome maturation. The amino acid asparagine was shown to specifically inhibit the fusion of autophagosomes with lysosomes, without inhibiting fusion with endosomes (Hoyvik *et al.*, 1991). The mechanisms of this inhibition are unknown. Inhibitors of lysosomal proteinases such as leupeptin inhibit degradation of segregated cytoplasm and cause accumulation of late autophagic vacuoles (Furuno *et al.*, 1982; Ishikawa *et al.*, 1983; Kovacs *et al.*, 1982). Endocytic tracer molecules still have access to autophagic vacuoles in leupeptin-treated cells, indicating that fusion with endosomes and possibly also with lysosomes takes place (Punnonen *et al.*, 1993). Bafilomycin A1 is a specific inhibitor of the lysosomal proton pump and, thus, it indirectly inhibits lysosomal enzymes, which have acidic pH optima. Interestingly, bafilomycin treatment was reported to inhibit fusion of autophagosomes with both endosomes and lysosomes (Mousavi *et al.*, 2001; Yamamoto *et al.*, 1998), suggesting that acidification of autophagic vacuoles, and/or endo/lysosomes, might be needed for the fusion.

3.2.2. Microtubules

The microtubule inhibitor vinblastine causes accumulation of mainly early autophagic vacuoles in hepatocytes (Hirsimaki and Pilstrom, 1982; Kovacs *et al.*, 1982), whereas mainly late autophagic vacuoles accumulate in fibroblasts (Miettinen and Reunanen, 1991). This drug inhibits the fusion of autophagosomes with lysosomes and probably also with endosomes (Berg *et al.*, 1998; Gordon and Seglen, 1988). Another microtubule inhibitor, nocodazole, causes accumulation of intermediate or late autophagic vacuoles in fibroblasts (Aplin *et al.*, 1992; Eskelinen *et al.*, 2002b). Thus it seems that disruption of microtubules causes accumulation of preferably early autophagic vacuoles in hepatocytes, whereas mainly late autophagic

vacuoles accumulate in fibroblastic cells treated with the drugs. Newer studies have also addressed the role of microtubules in autophagy. Tooze's group found that microtubules are needed for the fusion of autophagosomes with endosomes in primary rat hepatocytes. Surprisingly, they also report that microtubules facilitate autophagosome formation (Kochl *et al.*, 2006). Rubinsztein's group reported that the microtubule motor dynein has a role in the delivery of autophagosome contents to lysosomes (Ravikumar *et al.*, 2005). Elazar's group reported that autophagosomes, but not Atg5-positive phagophores, move along microtubules toward the microtubule-organizing center (Fass *et al.*, 2006). The authors propose that microtubules facilitate autophagosome formation and serve to direct them for degradation in lysosomes.

After their formation in the cytoplasm, autophagosomes travel on microtubules toward the microtubule-organizing center (Fass *et al.*, 2006) (Fig. 5.6B). Microtubules may also have a role in transporting autophagic machinery to the microtubule-organizing center in cells containing aggregated proteins. In cells containing aggregated huntingtin, protein aggregates, Atg proteins, and lysosomes were all transported to the cell center in a process that needed intact microtubules and the cytoplasmic deacetylase HDAC6 (Iwata *et al.*, 2005). The authors suggested that this transport enhanced the efficiency of aggregate segregation and formation of autolysosomes. In conclusion, it seems that intact microtubules serve two functions during macroautophagy: they assist autophagosome formation and delivery of segregated cargo to lysosomes for degradation.

3.2.3. Ubiquitin-activating enzyme E1

The ubiquitin-activating enzyme E1 is needed for the initiation of protein ubiquitination. Interestingly, autophagic maturation was disturbed in cells possessing a temperature-sensitive E1. When these cells were incubated at restrictive temperature (39°C), late autophagic vacuoles/autolysosomes accumulated. These autolysosomes were acidic and contained lysosomal enzymes (Lenk *et al.*, 1992). Unlike autophagic vacuoles observed in parental cells, these vacuoles did not contain ubiquitin-conjugated proteins. The authors proposed that protein ubiquitination may be required for their complete degradation in autolysosomes. In agreement with this proposal, ubiquitinated proteins have been shown to be enriched in the lysosomal compartment in fibroblasts (Laszlo *et al.*, 1990). Further, ubiquitinated proteins accumulate in cells deficient in Atg proteins (Komatsu *et al.*, 2005).

3.2.4. SKD1 and Hrs

The SKD1 AAA ATPase was shown to be necessary for autophagosome maturation (Nara *et al.*, 2002). SKD1 is a mouse homolog of the yeast Vps4 implicated in transport from endosomes to the vacuole. Disruption of SKD1 function led to disturbed endosome morphology and endosomal

transport (Yoshimori *et al.*, 2000). Autophagosomes accumulated in cells expressing the dominant negative forms of SKD1 (Nara *et al.*, 2002). By light microscopy, no delivery of a late endosomal marker to autophagosomes was detected in cells expressing the dominant negative SKD1. The authors concluded that fusion of autophagosomes with endosomes was blocked, and that this also prevented fusion with lysosomes. This implies that autophagosome maturation may be obligatorily stepwise, meaning that lysosomes are not able to fuse before endosomal vesicles have done so.

Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) is needed for the sorting of ubiquitinylated proteins into the internal vesicles of multivesicular endosomes (Gruenberg and Stenmark, 2004). Tamai and colleagues showed that autophagosome maturation was retarded in Hrs-depleted cells (Tamai *et al.*, 2007). It seems likely that Hrs function may be needed for the fusion of autophagic vacuoles with lysosomes, or for the final degradation of the segregated cytoplasmic cargo.

3.2.5. Vti1

In yeast, the SNARE protein Vti1 is needed for fusion of autophagosomes with the vacuole (Ishihara *et al.*, 2001). The mammalian homolog Vti1b may also have a role in autophagic vacuole maturation. Mainly late autophagic vacuoles accumulated in hepatocytes isolated from mice deficient in Vti1b (Atlashkin *et al.*, 2003), suggesting their maturation was partially retarded. Interestingly, fusion profiles, that is, vacuoles that seemed to be in the process of fusion, consisting of two autophagic vacuoles, as well as multivesicular endosomes were more prominent in the Vti1b-deficient hepatocytes. This suggests that Vti1b may be involved in completion of the fusion processes between autophagic vacuoles, or in fusion of multivesicular endosomes with autophagic vacuoles.

3.2.6. Rab7

The small GTPase Rab7 is required for autophagic vacuole maturation. Rab7 was shown to localize in the limiting membranes of autophagic vacuoles (Gutierrez *et al.*, 2004b; Jäger *et al.*, 2004). Immunogold labeling revealed that Rab7 was already present in AVi, although the labeling density increased during maturation to AVd (Jäger *et al.*, 2004). During starvation, autophagic vacuoles accumulated in cells where Rab7 function was inhibited by overexpression of dominant negative Rab7 or Rab7 RNA interference (Gutierrez *et al.*, 2004b; Jäger *et al.*, 2004), suggesting that Rab7 was required for their normal maturation. Quantitative electron microscopy showed that in Rab7 knockdown cells, the amount of AVi was not different from that in controls, but that the amount of AVd was increased. This suggests that the initial maturation steps leading to the formation of AVd was proceeding normally, but the final maturation of AVd was blocked. An endocytic tracer taken up to early endosomes was still delivered to

autophagic vacuoles in Rab7-inhibited cells, whereas delivery of a tracer taken up to lysosomes was inhibited (Gutierrez *et al.*, 2004b). Electron microscopy examination revealed that fusion of autophagic vacuoles with multivesicular endosomes was not affected in Rab7 knockdown cells (Eskelinen, 2005). Further, by electron microscopy the size of autophagic vacuole profiles was similar in control and Rab7 knockdown cells (Jäger *et al.*, 2004). This also suggests that initial fusion events with endosomes were occurring normally in the absence of Rab7. Both groups concluded that fusion of autophagosomes with endosomes still occurred in the absence of functional Rab7, but fusion with lysosomes was inhibited.

Another small GTP-binding protein, Rab24, was proposed to be associated with the formation of autophagosomes. During induction of autophagy by starvation, Rab24 redistributed from a perinuclear reticular localization to vesicles that also contained the autophagosome marker LC3 (Munafo and Colombo, 2002).

3.2.7. Lamp-2

Hepatocytes deficient in the lysosomal membrane protein LAMP-2 accumulated increased amounts of both early and late autophagic vacuoles (Tanaka *et al.*, 2000). An endocytic tracer was still delivered to autophagic vacuoles in the LAMP-2 knockout cells, and fusion with multivesicular endosomes was frequently detected (Eskelinen *et al.*, 2002a), indicating that fusion of autophagosomes with multivesicular endosomes was not disturbed, but fusion with lysosomes was probably inhibited. Starvation-induced degradation of long-lived proteins was retarded in the LAMP-2-deficient hepatocytes, in agreement with the autophagic maturation defect (Tanaka *et al.*, 2000). In addition to hepatocytes, autophagic accumulation was observed in certain other tissues including heart and skeletal muscle of the LAMP-2-deficient mice (Tanaka *et al.*, 2000). The phenotype of LAMP-2-deficient mice is similar to the symptoms described in patients suffering from Danon disease, a hereditary disease with lysosomal involvement leading to fatal cardiomyopathy (Nishino *et al.*, 2000). Danon disease is caused by mutations in the LAMP-2 gene.

Autophagy proceeded normally in fibroblasts deficient in LAMP-2. However, in fibroblasts deficient in both LAMP-2 and the structurally related LAMP-1, abnormal amounts of late autophagic vacuoles accumulated during starvation. Fusion events between autophagic vacuoles and multivesicular endosomes were frequently detected, suggesting that endosome fusion was not affected (Eskelinen *et al.*, 2004). The phenotype of LAMP-1/LAMP-2-deficient fibroblasts is similar to that of the Rab7 knockdown cells described previously (Jäger *et al.*, 2004). Taken together, we can conclude that fusion of autophagosomes with multivesicular endosomes takes place in both LAMP-2 single-deficient hepatocytes and LAMP-1/LAMP-2 double-deficient fibroblasts. AVis accumulate in excess

in LAMP-2-deficient hepatocytes, and AVds accumulate in both LAMP-2 single-deficient hepatocytes and LAMP double-deficient fibroblasts. The difference is possibly due to the different autophagy rates in these cell types: the autophagic pathway is much more active in hepatocytes than in fibroblasts. Alternatively, there may be differences in the expression levels of LAMP-1 between hepatocytes and fibroblasts. In conclusion, fusion of autophagic vacuoles with lysosomes seems to be retarded in hepatocytes lacking LAMP-2 and in fibroblasts lacking both LAMP-1 and LAMP-2. Similar maturation defects were observed in plasma membrane-derived phagosomes in LAMP double-deficient fibroblasts (Huynh *et al.*, 2007).

Interestingly, the delivery of Rab7 to autophagic vacuoles was retarded in the LAMP double-deficient fibroblasts (Jäger *et al.*, 2004). Even stronger defects in the delivery of Rab7 to phagosomes were observed in these cells (Huynh *et al.*, 2007). In addition to the accumulation of late autophagic vacuoles during starvation, the LAMP double-deficient cells also showed a prominent accumulation of unesterified cholesterol in endo/lysosomal Rab7-positive compartments (Eskelinen *et al.*, 2004). Lysosomal cholesterol accumulation has been proposed to interfere with Rab7 function (Lebrand *et al.*, 2002). However, clearance of the lysosomal cholesterol stores did not rescue the maturation of plasma membrane-derived phagosomes (Binker *et al.*, 2007; Huynh *et al.*, 2007), suggesting cholesterol storage was not the cause of the maturation defect.

3.2.8. Lysosomal cathepsins

As described previously, inhibition of lysosomal enzymes by drugs causes accumulation of late autophagic vacuoles. In agreement with these older findings, autophagic vacuoles were observed to accumulate in the brain and other tissues of 20-day-old mice deficient in the lysosomal proteinase cathepsin D (Koike *et al.*, 2000). Importantly, subunit c of mitochondrial ATP synthase was found to accumulate in the lysosomes of the cathepsin D-deficient neurons. It is likely that this protein was delivered into lysosomes via autophagy of mitochondria. Subunit c of ATP synthase is a component of ceroid lipofuscin that accumulates in neurons of patients suffering from a subclass of ceroid lipofuscinoses, a group of fatal neurodegenerative diseases (Tyynelä, 2004). In humans, this disease subclass is caused by mutations in CLN genes (numbers 2, 3, 4, 5, 6, 7, and 8), encoding either the lysosomal enzyme tripeptidyl peptidase I or a group of novel lysosomal membrane proteins. The rest of the neuronal ceroid lipofuscinoses are caused by mutations in the CLN1 gene, encoding the enzyme palmitoyl protein thioesterase. Instead of the mitochondrial ATPase subunit c, saposins accumulate in neuronal lysosomes of these patients. Cathepsin D-deficient mice show a similar phenotype to that of patients suffering from accumulation of subunit c, and it is likely that mutations in cathepsin D will also be detected in human patients in future.

It should be noted that, although starvation-responsive autophagy is low or absent in the brain of adult mice (Mizushima *et al.*, 2004), autophagy has a role in neurons during brain development or in normal housekeeping (see Section 4.1.4). Mice double deficient in cathepsins B and L were shown to suffer from neuronal loss and brain atrophy (Felbor *et al.*, 2002). By electron microscopy, the neurons were observed to accumulate vesicles that resembled late autophagic vacuoles. Taken together, the findings with cathepsin-deficient mice indicate that cathepsins D, B, and L are necessary for degradation of autophagocytosed material in neurons. Disturbing the degradation of autophagocytosed cytoplasm leads to disturbed function of neurons, and to disease.

3.2.9. Presenilin 1

Presenilin 1 is best known for its role in the intramembrane proteolysis of the amyloid precursor protein, which leads to liberation of the amyloid β peptide. Overproduction of this peptide drives the formation of amyloid in Alzheimer's disease. Presenilin 1 is the catalytic component of the γ-secretase complex that consists of nicastrin, Pen-2, and Aph-1 proteins (de Strooper, 2003). Vacuoles resembling late autophagic vacuoles were shown to accumulate in presenilin 1-deficient mixed cortical neurons (that were positive for α-synuclein) and in presenilin 1- and presenilin 2-deficient mouse embryonic fibroblasts (Wilson *et al.*, 2004). Another group (Esselens *et al.*, 2004) observed that presenilin 1-deficient primary hippocampal neurons accumulated telencephalin, an intercellular cell adhesion molecule that interacts with presenilin 1 (Annaert *et al.*, 2001), in large vacuoles. These vacuoles were also positive for LC3 and Atg12, but devoid of endo/lysosomal markers. Using cathepsin D-deficient neurons, the researchers confirmed that telencephalin was normally degraded via autophagy. These findings were interpreted to suggest an involvement of presenilin 1 in the autophagic process, possibly in the maturation of autophagic vacuoles. The interpretation is in line with endosomal/lysosomal abnormalities observed in the brain of Alzheimer's disease patients (Nixon *et al.*, 2000). Interestingly, accumulation of degradative organelles in presenilin-deficient neurons could be suppressed by exogenous expression of dominant negative presenilin 1 mutants, indicating that the putative role of presenilin 1 in autophagy was not dependent on its well-documented γ-secretase activity (Esselens *et al.*, 2004; Wilson *et al.*, 2004). However, the exact molecular mechanisms underlying this new presenilin 1 function remain to be elucidated. In this respect it is worth mentioning that although presenilin 1 is mainly localized in pre-Golgi compartments (Annaert *et al.*, 1999), small amounts are localized in endosomal/lysosomal fractions (Pasternak *et al.*, 2003; Rechards *et al.*, 2003).

Neuronal autophagy was shown to be induced early in Alzheimer's disease, and before β-amyloid deposited extracellularly in the presenilin

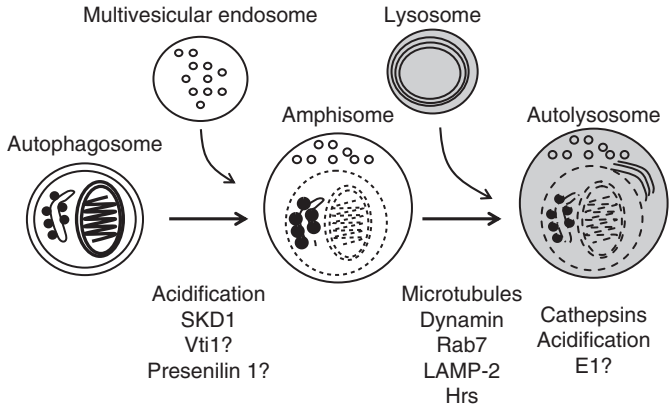

Figure 5.9 A summary of factors that regulate the fusion steps during autophagosome maturation. Cathepsins, acidification, and E1 are needed for degradation in autolysosomes.

1/amyloid precursor protein mouse model of β-amyloidosis. Autophagosomes and late autophagic vacuoles accumulated in dystrophic dendrites, suggesting an impaired maturation of autophagic vacuoles. Immunolabeling identified autophagic vacuoles in the brain as a major reservoir of intracellular β-amyloid (Yu *et al.*, 2005). The authors suggested that impaired maturation of autophagic vacuoles, caused by mutations in presenilins, can lead to increased formation of β-amyloid in these vacuoles. A summary on factors participating in autophagosome maturation is presented in Fig. 5.9.

4. Functions and Regulation of Autophagy

4.1. Functions

4.1.1. Survival mechanism during short-term starvation

Autophagy is a stress response. The role of autophagy as a survival mechanism during short-term amino acid starvation is well documented. The best known inducer of macroautophagy in mammalian cells is starvation of serum and amino acids. Autophagosomes can already be detected after 15–30 min of starvation (Kabeya *et al.*, 2000). When measured by quantitative electron microscopy, the level of autophagic vacuoles reaches a steady state level in 2–4 h, and then it starts to decrease (Eskelinen *et al.*, 2002b). During long-term starvation, chaperone-mediated autophagy increases and macroautophagy decreases (Cuervo and Dice, 1998; Fuertes *et al.*, 2003). This way the cell can prevent wasting too much of its cytoplasm via the nonselective macroautophagic pathway. In cultured animal cells, starvation

of serum, but not amino acids, also increases the cytoplasmic volume fraction of autophagic vacuoles, but less than starvation of both serum and amino acids (Eskelinen *et al.*, 2002b). Yeast strains defective in autophagy do not survive starvation of a nitrogen source (Klionsky, 2004). Knockout mice deficient in one of the autophagy proteins, Atg5, showed that autophagy is indispensable for the energy metabolism of mice immediately after birth (Kuma *et al.*, 2004). Atg5 knockout mice died of starvation 1 day after birth.

4.1.2. Organelle turnover

In muscle and heart cells, autophagy seems to have a special housekeeping role in the turnover of cytoplasmic constituents including mitochondria. This is revealed by myopathy and cardiomyopathy in patients and mice with defective autophagic degradation due to deficiency of the lysosomal membrane protein LAMP-2 (Nishino, 2003; Nishino *et al.*, 2000; Tanaka *et al.*, 2000). Large vacuoles containing partially degraded cytoplasm accumulate in LAMP-2-deficient heart and skeletal muscle. In LAMP-2-deficient mice, cardiomyocytes showed accumulation of mitochondria-containing autophagosomes (Stypmann *et al.*, 2006), suggesting that autophagy is important for mitochondrial turnover. The importance of autophagy for the heart muscle is supported by a study showing that heart-specific loss of the autophagy protein Atg5 caused cardiomyopathy in mice (Nakai *et al.*, 2007). Studies have suggested that damaged mitochondria might be autophagocytosed selectively in a process termed mitophagy (see Section 1) (Kim *et al.*, 2007). Mitochondria are the major source of reactive oxygen species in cells. Interestingly, reactive oxygen species were shown to be necessary for the signal transduction pathway initiating starvation-induced autophagy (Scherz-Shouval *et al.*, 2007).

4.1.3. Growth regulation and cancer

In addition, autophagy has a role in growth regulation, as suggested by decreased autophagy during growth of the kidney after unilateral nephrectomy (Jurilj and Pfeifer, 1990). Inducible knockdown of the autophagy protein Atg5 showed that autophagy negatively controls cell size (Hosokawa *et al.*, 2006). A similar result was shown in the *Drosophila* fat body with overexpression of autophagy protein Atg1 (Scott *et al.*, 2007).

Impaired autophagy has been proposed to contribute to cancer development. Beclin 1, a mammalian homolog of the yeast autophagy protein Atg6/Vps30, is monoallelically deleted in a large proportion of human breast and ovarian cancers. Overexpression of Beclin 1 in a breast cancer cell line increased autophagy and decreased the growth and tumorigenicity of these cells (Liang *et al.*, 1999). Further, mice with heterozygous deletion of Beclin 1 had less autophagy and more tumors than control mice (Qu *et al.*, 2003; Yue *et al.*, 2003). Impaired autophagy could contribute to

tumorigenesis via impaired regulation of cell growth, and/or via decreased cell death (see Section 4.1.7). Other alternatives also exist. One study showed that failure to sustain metabolism via autophagy resulted in increased DNA damage. This chromosomal instability was proposed to increase tumor progression (Mathew *et al.*, 2007).

4.1.4. Turnover of aggregate-prone proteins

Autophagy contributes to intracellular quality control and housekeeping, especially in turnover of aggregate-prone proteins. Prevention of autophagy by conditional knockout led to accumulation of ubiquitinated protein aggregates in mouse tissues (Komatsu *et al.*, 2005). Tissue-specific knockout of autophagy proteins in the central nervous system caused accumulation of ubiquitin-positive protein aggregates and neurodegeneration in mice (Hara *et al.*, 2006; Komatsu *et al.*, 2006). Further, autophagy was shown to reduce the toxicity of protein aggregates in Huntington disease (Ravikumar *et al.*, 2004), which may have important therapeutic implications. Autophagy may prevent aggregate formation by degrading the proteins as monomers, oligomers, or after aggregate formation (Klionsky, 2006). It is not clear at present whether aggregated proteins are segregated preferentially, or whether they are removed via unspecific autophagic uptake of cytoplasm. Two proteins have been proposed to function during the uptake of protein aggregates: Alfy and p62 (Bjorkoy *et al.*, 2006; Simonsen *et al.*, 2004).

In addition to removal of cytoplasmic aggregate-prone proteins, autophagy also contributes to quality control in the endoplasmic reticulum (ER). Results have demonstrated that the unfolded protein response induces autophagy, and that this induction is beneficial for cell survival (Ding *et al.*, 2007; Ogata *et al.*, 2006; Yorimitsu *et al.*, 2006).

4.1.5. Host defense against intracellular pathogens

In some cases autophagy can protect cells against intracellular pathogens. Induction of autophagy during herpes simplex viral infection and localization of viral particles inside autophagic vacuoles was proposed to indicate that autophagy acts as a host defense mechanism in infected cells (Talloczy *et al.*, 2002). Interestingly, the herpesvirus virulence protein ICP34.5 was shown to inhibit autophagy, suggesting that the virus has developed a way to prevent the autophagic defense of the host cell. Autophagy may also help cells to defend against some intracellular bacteria (Rich *et al.*, 2003). Sequestration of intracellular group A streptococci in autophagosome-like structures was proposed to protect cells against the bacteria (Nakagawa *et al.*, 2004). *Mycobacterium tuberculosis* is normally able to survive inside macrophages by preventing the fusion of phagosomes with lysosomes. Surprisingly, induction of autophagy was shown to bypass the maturation defect, leading to phagolysosome formation and bacterial killing (Gutierrez *et al.*, 2004a).

4.1.6. Antigen presentation

Macroautophagy also contributes to antigen presentation. Major histocompatibility complex (MHC) class II molecules present products of lysosomal proteolysis to $CD4^+$ T cells. Extracellular antigen uptake is considered to be the main source of MHC class II ligands. However, it was demonstrated that in MHC class II-positive cells, including dendritic cells, B cells, and epithelial cells, autophagosomes continuously fused with multivesicular MHC class II-loading compartments (Schmid *et al.*, 2007). This pathway was of functional relevance, because targeting of the influenza matrix protein 1 to autophagosomes enhanced its MHC class II presentation to $CD4^+$ T cells. It was suggested that macroautophagy efficiently delivers cytosolic proteins for MHC class II presentation and can improve helper T cell stimulation.

4.1.7. Programmed cell death

Surprisingly, autophagy also seems to have roles in programmed cell death. Type II programmed cell death, or autophagic cell death, was originally described in mammary carcinoma cells (Bursch, 2001; Bursch *et al.*, 1996). The so-called death-associated protein kinase (DAPk), which can act as a tumor suppressor, may function in the signaling pathway that links autophagy to cell death (Inbal *et al.*, 2002). Autophagy proteins have been shown to be necessary for certain types of cell death (Pyo *et al.*, 2005; Shimizu *et al.*, 2004; Yu *et al.*, 2004). In this scenario autophagy is needed for the execution of cell death. Under other conditions, such as nutrient starvation, autophagy may protect cells against apoptosis by providing nutrients (Bauvy *et al.*, 2001; Boya *et al.*, 2005; Gonzales-Polo *et al.*, 2005).

The regulation of apoptosis and autophagy was shown to be linked via the antiapoptotic protein Bcl-2. Bcl-2 was shown to inhibit Beclin 1-dependent autophagy by binding to Beclin 1 and preventing its association with Vps34 (Pattingre *et al.*, 2005). This antiautophagy function of Bcl-2 was proposed to help maintain autophagy at levels that are compatible with cell survival, rather than cell death. Lipids may also regulate autophagy and its outcome to the host cell. Ceramide and sphingosine 1-phosphate, a metabolite of ceramide, both induce autophagy in mammalian cells (Lavieu *et al.*, 2006). The outcome on cell survival is, however, different: ceramide promotes cell death, whereas sphingosine 1-phosphate increases cell survival. Ceramide was part of the signaling cascade initiated by chemotherapy, whereas sphingosine 1-phosphate was part of the signaling cascade initiated by starvation. The level of Beclin 1 was higher during ceramide signaling, and the autophagy response was also stronger (Lavieu *et al.*, 2007).

One study showed yet another function for autophagy during embryonic development. Autophagy genes were shown to be necessary for the clearance of apoptotic cells during embryonic development in mice (Qu *et al.*, 2007). Autophagy was suggested to contribute to the clearance of dead

cells during programmed cell death by maintaining cellular energy levels, thereby allowing the generation of cell surface and secreted signals that then promote engulfment of cell corpses by neighboring cells.

4.1.8. Longevity

Finally, autophagy may also contribute to longevity. Long-term reduced caloric intake increases longevity in several animal species. Evidence suggests that increased autophagic turnover of cytoplasmic constituents including mitochondria may contribute to the longer life in the long-term dieting animals (Bergamini *et al.*, 2003). Further evidence that autophagy contributes to longevity came from *Caenorhabditis elegans* mutants possessing a defective insulin receptor (daf2 mutant), which live longer than control worms. The increased lifetime of these mutant worms was shown to depend on a functional autophagic pathway (Melendez *et al.*, 2003). Knockdown of other autophagy gene products including Atg7 and Atg12 was shown to shorten the life span of both wild-type and daf2 mutant *C. elegans* (Hars *et al.*, 2007). Together these studies strongly support a role for autophagy in the regulation of aging.

4.2. Regulation

Autophagy is a tightly regulated process. Too little autophagy can be lethal, especially during nutrient starvation, and too much autophagy can lead to cell demise. Availability of nutrients, amino acids in particular, is the most important physiological regulator of macroautophagy. Signaling pathways participating in the nutrient control of autophagy in mammalian cells have been reviewed (Codogno and Meijer, 2005; Kadowaki *et al.*, 2006; Meijer and Codogno, 2006). A description on the most important regulators is presented here. Figure 5.10 presents a summary of these regulatory pathways.

4.2.1. Amino acids

Amino acid deficiency induces autophagy, and autophagy is inhibited by amino acids. Thus amino acids generated by degradation of autophagocytosed proteins act as a feedback inhibitor of autophagosome formation. Two amino acid sensors have been described in autophagy signaling: the protein kinase Gcn2, which is activated by uncharged transfer RNA molecules (Talloczy *et al.*, 2002), and an amino acid receptor located on the plasma membrane (Kanazawa *et al.*, 2003). Gcn2 senses intracellular amino acid levels, whereas the plasma membrane amino acid receptor senses extracellular amino acids. The signals from Gcn2 to autophagy are mediated by a pathway including the eukaryotic initiation factor eIF2α, which supports autophagy when phosphorylated at Ser-51 (Talloczy *et al.*, 2002). eIF2α could thus act as a switch between protein synthesis (in the presence of amino acids) and autophagic protein degradation (in the absence of amino acids).

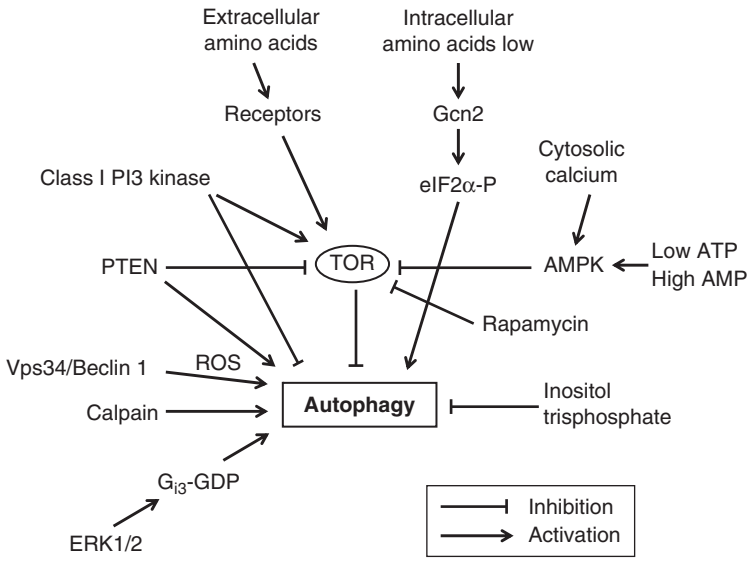

Figure 5.10 A summary of signaling pathways and factors regulating autophagy. Further details are described in text.

The putative amino acid receptor at the plasma membrane seems to mediate the signals from an extracellular nontransportable leucine analog to the autophagic pathway (Kanazawa *et al.*, 2003), possibly via target of rapamycin (TOR) kinase. Future studies will show whether both suggested amino acid sensors are active in the same cells, and whether different amino acids use different signal transduction pathways to regulate autophagy and protein synthesis.

As mentioned previously, reactive oxygen species (ROS) were shown to be necessary for autophagy induced by starvation. ROS were shown to inhibit the delipidating enzyme Atg4, thus allowing lipidation of LC3 that is needed for autophagosome formation (Scherz-Shouval *et al.*, 2007). The authors suggest that ROS act downstream of the Beclin 1–Vps34 complex in the signal transduction pathway leading to autophagosome formation.

4.2.2. TOR kinase and Atg1/ULK1

Target of rapamycin (TOR) protein kinases play a conserved role in nutrient sensing, regulation of growth and proliferation, and control of a wide variety of metabolic processes. In yeast, TOR plays a crucial role in autophagy regulation, which is in line with its role as a nutrient sensor. Active TOR kinase inhibits autophagy, and inhibition of the kinase activity by rapamycin activates autophagy (Kamada *et al.*, 2000). The serine/threonine kinase Atg1 was shown to act as a link between TOR signaling and autophagy.

Interaction of Atg1 and Atg13 is regulated in such a way that during high TOR activity Atg13 is hyperphosphorylated and not associated with Atg1. Starvation or TOR inhibition with rapamycin results in decreased phosphorylation of Atg13, increased assembly of Atg1–Atg13 complexes, and increased autophagosome formation (Kamada *et al.*, 2000). In mammalian cells, TOR inhibition by rapamycin also induces autophagy, although this induction is weaker than that by amino acid starvation (Blommaart *et al.*, 1995). Atg1/ULK1 is essential for autophagy induction in *Drosophila* and mammalian cells (Scott *et al.*, 2004; Young *et al.*, 2006). ULK1 acts downstream of TOR also in mammalian cells (Chan *et al.*, 2007), but it is not known how closely the signaling pathway resembles that of yeast signaling. It is possible that in mammals, some regulatory pathways such as insulin include TOR, whereas others such as amino acids do not (Kanazawa *et al.*, 2003).

Autophagy is also energy dependent. ATP is required for autophagosome formation, fusion of autophagosomes with lysosomes, and lysosomal degradation (Plomp *et al.*, 1989). TOR was proposed to act as a sensor for intracellular ATP (Dennis *et al.*, 2001). AMP-activated protein kinase was reported to mediate the ATP sensing of TOR (Meijer and Dubbelhuis, 2004). Thus it is possible that ATP levels control autophagy directly and/or via TOR signaling (Meijer and Codogno, 2007).

4.2.3. Phosphatidylinositol 3-kinases

Phosphatidylinositol 3-kinases (PI3-kinases) also regulate autophagy. As discussed previously, the activity of class III PI3-kinase Vps34 is absolutely required for autophagosome formation (Eskelinen *et al.*, 2002b; Petiot *et al.*, 2000) and accordingly this process is completely inhibited by PI3-kinase inhibitors including 3-methyladenine (Seglen and Gordon, 1982), wortmannin and LY294002 (Blommaart *et al.*, 1997), as well as microinjection of inhibitory antibodies against Vps34 (Eskelinen *et al.*, 2002b).

Interestingly, class I PI3-kinases have an opposite effect on autophagy: the activation of these kinases is inhibitory (Petiot *et al.*, 2000). In line with this, PTEN (phosphatase and tensin homolog), a tumor suppressor and phosphatase that decreases the concentration of class I PI3-kinase product, enhances autophagy (Arico *et al.*, 2001). It is not known whether the signaling from class I PI3-kinases to autophagy proceeds via TOR, and/or a parallel pathway. Because the activities of class I PI3-kinases promote cell growth and these kinases are upregulated in many cancers, it is possible that this signaling pathway might be linked to the role of autophagy in growth control.

4.2.4. Trimeric G proteins

Trimeric G proteins regulate autophagosome formation, trimeric G_{i3} protein in particular. Autophagy is active when G_{i3} is bound to GDP and inactive when it is bound to GTP. In addition, G_{i3} must be associated with

intracellular membranes, the Golgi apparatus, or the endoplasmic reticulum to control autophagosome formation (Codogno and Meijer, 2004). G_α-interacting protein (GAIP) belongs to the protein family of regulators of G protein signaling. GAIP favors autophagosome formation by accelerating GTP hydrolysis by the $G_{\alpha i3}$ protein. Serine phosphorylation of GAIP stimulates its GTPase-enhancing activity, and consequently the autophagic pathway (Ogier-Denis *et al.*, 2000). This serine phosphorylation is dependent on the activity of mitogen-activated protein (MAP) kinases ERK1/2 (extracellular signal-regulated kinases 1/2), which are turned off in the presence of amino acids (Pattingre *et al.*, 2003). Thus there seems to be a link between amino acid signaling and control of autophagy by trimeric G proteins (Codogno and Meijer, 2004).

4.2.5. Other regulatory factors

Free cytosolic calcium was reported to control macroautophagy: a rise in cytosolic calcium induced autophagy (Hoyer-Hansen *et al.*, 2007). Several calcium-mobilizing agents including vitamin D_3 and ATP were shown to inhibit TOR, suggesting free calcium functions upstream of TOR in autophagy signaling. This signaling was mediated by calcium/calmodulin-dependent kinase kinase-β and AMP-activated protein kinase, and inhibited by expression of endoplasmic reticulum-targeted Bcl-2.

Ubiquitously expressed μ- and m-calpain proteases consist of 80-kDa catalytic subunits Capn1 and Capn2, and a common 28-kDa regulatory subunit, Capns1. Autophagy was shown to be impaired in cells deficient in Capns1 (Demarchi *et al.*, 2006). The authors suggest that calpain could modulate one or more components of the signaling networks involved in autophagosome formation. Another possibility could be the involvement of calpain in the processing of cytoskeletal connections that were shown to be necessary for autophagosome formation (see previously) (Demarchi *et al.*, 2007).

Another autophagy regulator is inositol trisphosphate. Lithium was reported to induce autophagy. The autophagy-enhancing properties of lithium were mediated by inhibition of inositol monophosphatase and led to free inositol depletion. This, in turn, decreased *myo*-inositol 1,4,5-triphosphate (IP3) levels (Sarkar *et al.*, 2005). Also, another group reported that reduction of intracellular IP3 levels stimulated autophagy, whereas increased levels were inhibitory during starvation-induced autophagy (Criollo *et al.*, 2007). Knockdown of the IP3 receptor also stimulated autophagy. In addition, autophagy induced by starvation or IP3 receptor blockade was inhibited by Bcl-2 and Bcl-X_L targeted to the endoplasmic reticulum. Importantly, autophagy triggered by IP3 receptor inhibition was not modulated by calcium levels in the endoplasmic reticulum or cytosol, suggesting this signaling is not part of the cascade including cytosolic calcium and TOR.

5. Concluding Remarks

The initial description of autophagy in mammalian cells was published by De Duve (1969). Now we finally have specific marker proteins and genetic tools to elucidate the mechanisms and functions of this fascinating cellular process. Many new mammalian homologs of the yeast autophagy genes are likely to be identified in the near future. This will further assist in identifying autophagic structures and in correctly estimating the autophagic activity in cell lines and tissues. The known physiological roles and functions of autophagy are likely to increase in number and diversity.

In the past, autophagy was considered a "destructive" pathway. New results, however, point to a protective role, because disturbed autophagy leads to disease. Further, results suggest that induction of autophagy may protect against cancer (Qu *et al.*, 2003; Yue *et al.*, 2003) or even extend the life span (Hars *et al.*, 2007; Melendez *et al.*, 2003).

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