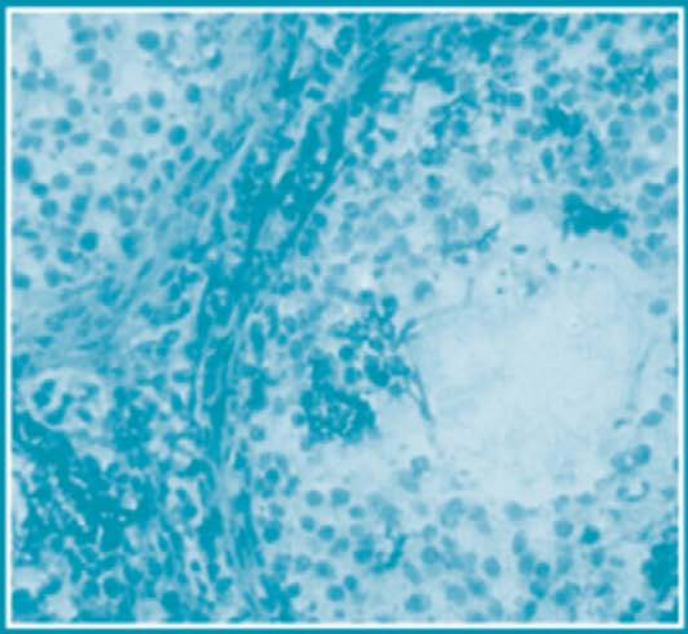
INTERNATIONAL REVIEW OF CYTOLOGY

A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



Volume 212

ACADEMIC PRESS

International Review of Cytology
A Survey of Cell Biology

VOLUME 212

SERIES EDITORS

Geoffrey H. Bourne	**1949–1988**
James F. Danielli	**1949–1984**
Kwang W. Jeon	**1967–**
Martin Friedlander	**1984–1992**
Jonathan Jarvik	**1993–1995**

EDITORIAL ADVISORY BOARD

International Review of Cytology
A Survey of Cell Biology

Edited by

Kwang W. Jeon
Department of Biochemistry
University of Tennessee
Knoxville, Tennessee

VOLUME 212

ACADEMIC PRESS
An Elsevier Science Imprint
San Diego San Francisco New York Boston London Sydney Tokyo

Front cover photograph: Immunochemical staining of macrophage populations in marine tissues of the ovary with the antiscavenger receptor class A monoclonal antibody 2F8. (For more details, see color insert figure 1.5.)

This book is printed on acid-free paper. ∞

Academic Press
An Elsevier Science Imprint.
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.academicpress.com

Academic Press
32 Jamestown Road, London NW1 7BY, UK
http://www.academicpress.com

International Standard Book Number: 0-12-364616-2

PRINTED IN THE UNITED STATES OF AMERICA
01 02 03 04 05 06 EB 9 8 7 6 5 4 3 2 1

CONTENTS

G Protein-Coupled Receptors: Dominant Players in Cell–Cell Communication

Joël Bockaert, Sylvie Claeysen, Carine Bécamel, Sylvie Pinloche, and Aline Dumuis

Genes Involved in the Initiation of DNA Replication in Yeast

Arturo Calzada and Avelino Bueno

Phenotype Switching in Polymorphic *Tetrahymena:* A Single-Cell Jekyll and Hyde

Phillip E. Ryals, Harriett E. Smith-Somerville, and Howard E. Buhse, Jr.

Mitosis and Motor Proteins in the Filamentous Ascomycete, *Nectria haematococca,* and Some Related Fungi

James R. Aist

This Page Intentionally Left Blank

CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

James R. Aist (239), *Department of Plant Pathology, College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853*

Peter W. Baas (41), *Department of Neurobiology and Anatomy, MCP Hahnemann University, Philadelphia, Pennsylvania 19129*

Carine Bécamel (63), *UPR CNRS 9023, 34094 Montpellier Cedex 5, France*

Joël Bockaert (63), *UPR CNRS 9023, 34094 Montpellier Cedex 5, France*

Avelino Bueno (133), *Instituto de Microbiología—Bioquímica/Centro de Investigación del Cáncer, Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno, CSIC/Universidad de Salamanca, 37007 Salamanca, Spain*

Howard E. Buhse, Jr. (209), *Department of Biological Sciences, University of IIIinois, Chicago, IIIinois 60607*

Arturo Calzada (133), *Instituto de Microbiología—Bioquímica/Centro de Investigación del Cáncer, Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno, CSIC/Universidad de Salamanca, 37007 Salamanca, Spain*

Sylvie Claeysen (63), *UPR CNRS 9023, 34094 Montpellier Cedex 5, France*

Liz Darley (1), *Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom*

Aline Dumuis (63), *UPR CNRS 9023, 34094 Montpellier Cedex 5, France*

Siamon Gordon (1), *Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom*

Richard Haworth (1), *Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom*

Nick Platt (1), *Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom*

Sylvie Pinloche (63), *LIRMM, 34392 Montpellier Cedex 5, France*

Phillip E. Ryals (209), *Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, Mississippi 39762*

Harriett E. Smith-Somerville (209), *Department of Biological Sciences and Coalition for Biomolecular Products, University of Alabama, Tuscaloosa, Alabama 35487*

The Many Roles of the Class A Macrophage Scavenger Receptor

Nick Platt, Richard Haworth, Liz Darley, and Siamon Gordon
Sir William Dunn School of Pathology, University of Oxford,
Oxford OX1 3RE, United Kingdom

The class A macrophage scavenger receptor (SR-A) is the prototypic example of a group of plasma membrane receptors collectively known as scavenger receptors. SR-A displays the ability to bind and endocytose large quantities of modified lipoprotein. Hence, it is thought to be one of the main receptors involved in mediating lipid influx into macrophages (Mϕ), which promotes their conversion into foam cells that are abundant in the atherosclerotic lesion. However, as a result of increased interest and research effort and through the development of specific reagents and animal models, it is now appreciated to be multifunctional. These roles include Mϕ growth and maintenance, adhesion to the substratum, cell–cell interactions, phagocytosis, and host defense. In this review, we attempt to summarize the evidence and argue that these kinds of activities underlie the biological versatility of Mϕ.

KEY WORDS: Scavenger receptor, Macrophage, Atherosclerosis, Innate immunity, Phagocytosis, Endocytosis, Pattern recognition, Lipopolysaccharide, Apoptosis.

I. Introduction

Mononuclear phagocytes are best known for their contribution to protection of the host against infection. The pioneering observations of Metchnikoff and others in the late 19th century described the recruitment of phagocytes to sites of infection and injury and their ability to kill invading microorganisms (Stossel, 1999). In subsequent years, the growing research interest in professional phagocytes, the neutrophil and the macrophage (Mϕ), has indicated that they have important

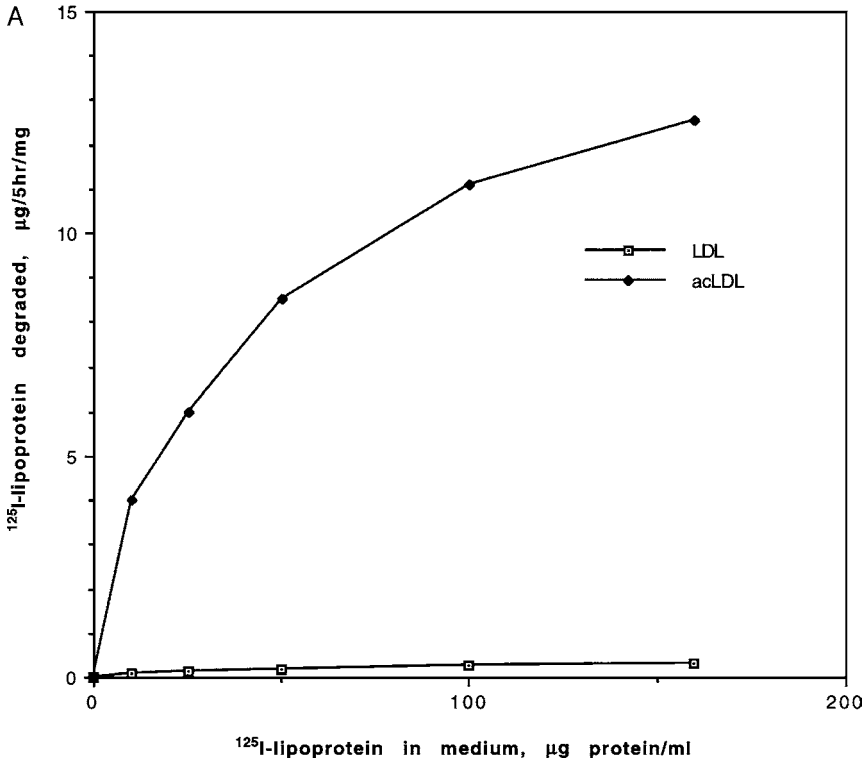


FIG. 1 (A) Degradation of ^{125}I-LDL or ^{125}I-acLDL by murine peritoneal macrophages. (B) Inhibition of macrophage-mediated degradation of ^{125}I-acLDL by competing LDL or maleyl-LDL (redrawn with permission from Goldstein *et al.,* 1979).

functions in addition to phagocytosis of pathogens. Plasma membrane receptors that enable the Mϕ to sense the complex, changing environment in which they reside are required for these activities. These surface receptors may, for example, permit the uptake of soluble molecules and particles or transduce extracellular signals that evoke particular responses from the cell. It would therefore be necessary for the Mϕ to express a plethora of different receptors, each dedicated to a specific cellular activity. However, an emerging trend in our understanding is that a single molecular species may not be involved in only one biological process but may be exploited in several distinct situations. In this article, we review the evidence that supports multiple activities for the class A macrophage scavenger receptor (SR-A) and the molecular basis of this property.

Macrophages are known as "professional phagocytes" or "scavengers" because of their propensity for phagocytosis, but the term "scavenger receptor" was initially defined in relation to the endocytic capacity of Mϕ for lipoproteins. Brown and

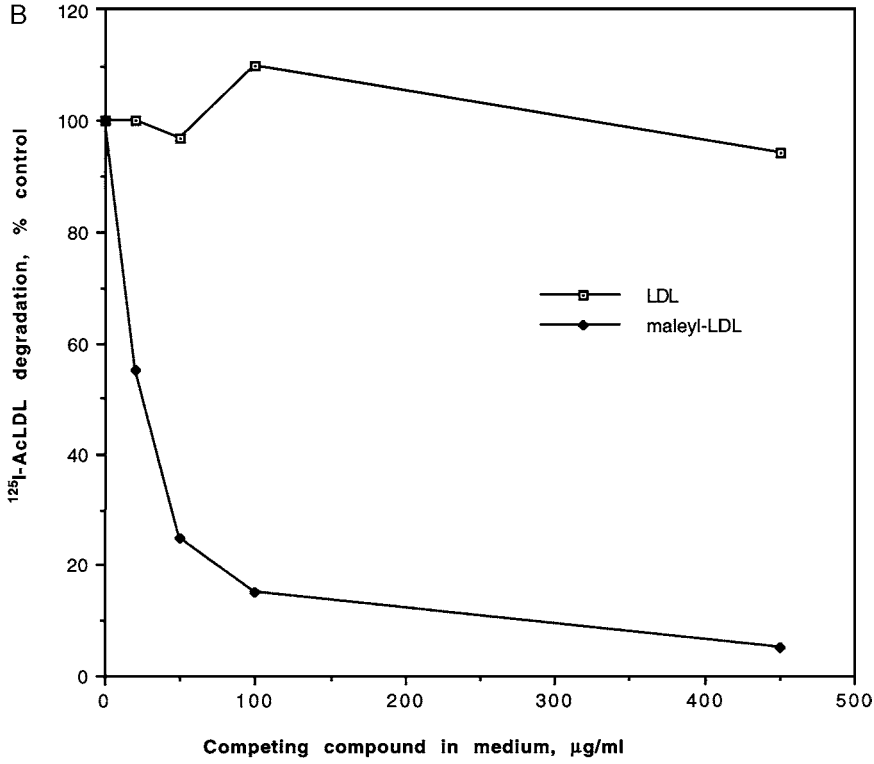


FIG. 1 *(continued)*

Goldstein (1983) demonstrated the existence of receptor-mediated mechanisms for the uptake of large quantities of chemically modified low-density lipoprotein (LDL) by Mϕ (Fig. 1). Originally known as acetylated LDL (acLDL) receptors, they are now termed scavenger receptors (SRs). The culmination of this high-capacity endocytosis is the conversion of Mϕ into cells that are packed with esterified cholesterol that resemble foam cells of the atherosclerotic plaque. Thus, SRs appear to play a central role in atherogenesis, but their exact contribution remains to be resolved and is the major focus of ongoing research on these receptors. However, we do not aim to discuss the relationship between SRs and atherosclerosis in detail, for a review of this subject, see de Winther *et al.* (2000).

The continuing exploration of lipoprotein metabolism has confirmed that a single receptor is not responsible for the endocytosis of all modified lipoprotein. There is now known to be a family of molecules that are collectively known as SRs. They are related by biological property rather than by sequence homology and are categorized into at least five classes (Pearson, 1996; Platt and Gordon, 1998). Although all SRs behave in similar ways, each has distinctive characteristics that

distinguish it from other members of the family. We concentrate on studies concerned with class A SR types SR-AI and SR-AII. Different SRs, notably the class B receptors SR-B1 (Krieger, 1999) and CD36 (Greenwalt *et al.,* 1992), have been the subjects of other recent reviews.

II. Biochemistry and Molecular Biology of SR-A

A. Isolation and Structure of SR-A

The acLDL receptor, known as the class A scavenger receptor, was originally purified by Kodama and colleagues (1988) through a scheme involving affinity chromatography of an extract of bovine lung. They partially purified a protein with acLDL binding activity and used the material to generate a specific monoclonal antibody (mAb) and in turn used this reagent to isolate to near homogeneity the 200-kDa protein. Gel electrophoresis of the immunoprecipitate under reducing conditions converted it to a single band of 77 kDa, suggesting the receptor is composed of three identical subunits. Digestion with sialidase and endoglycosidase F further increased its mobility, indicating the presence of asparagine-linked oligosaccharides and sialic acid. The sequences of two peptides derived by cyanogen bromide digestion were determined and a pool of degenerate oligonucleotides was designed and used to screen a bovine lung cDNA library (Kodama *et al.,* 1990). Initially, five clones were obtained and found to contain the predicted peptide sequences and were subsequently used to isolate a series of overlapping clones. Sequencing revealed a collagen-like triple helix that had not previously been observed in an integral membrane protein. When transiently expressed in COS cells, the clone could mediate internalization and degradation of ^{125}I-labeled acLDL. Subsequently, cDNAs encoding SR-A have been isolated from mouse, human, and rabbit (Krieger and Herz, 1994).

SR-A is a trimeric integral membrane glycoprotein that exists in two forms, types I and II, which are generated by alternative splicing of a single gene product (Fig. 2, see color insert) (Freeman *et al.,* 1990; Kodama *et al.,* 1990; Rohrer *et al.,* 1990). The two class A SRs, types I and II, comprise six domains including an extracellular α-helical coiled coil and collagenous region, which oligomerize to form a trimeric molecule. This collagenous domain is characteristic of the class A SRs and is present in the other member of the class that has been identified, the (MARCO) molecule (Elomaa *et al.,* 1995; Kodama *et al.,* 1996). Of the six SR-A domains that have been defined, domains I–V are shared by the two receptor types, whereas domain VI is restricted to the type I receptor. The 1457-bp murine type I receptor cDNA encodes a protein of 454 amino acids with a predicted mass of 49.6 kDa, whereas a 4-kb transcript of the type II receptor cDNA encodes a 350-amino-acid molecule with a predicted mass of 38.2 kDa. The two sequences diverge at residue 348, at which the type II cDNA encodes 3 additional amino acids to complete the

6-amino-acid C-terminal domain. A long 3′ untranslated region follows these nine nucleotides. The main features of each of the domains are as follows:

Domain I (residues 1–51 of the murine sequence) comprises the SR-A cytoplasmic tail that contains the N-terminal part of the polypeptide, which orientates the protein as a type II transmembrane molecule. The cytoplasmic tail does not contain any of the classical motifs that are present in other endocytic receptors, such as the transferrin receptor (Jing *et al.,* 1990). There are three conserved potential phosphorylation sites, two of which have the consensus motif for protein kinase C and one for calmodulin-dependent protein kinase II. There is species divergence in the N-terminal region of the cytoplasmic tail, which implies that it does not play a critical role in directing receptor-mediated endocytosis.

Domain II is the transmembrane domain (26–28 hydrophobic residues) and contains a conserved proline at position 67, which may affect the packing of trimers in the membrane.

Domain III is the spacer domain and has two of the seven potential N-linked glycosylation sites.

Domain IV forms an α-helical coiled coil structure on the basis of 23 heptad repeats. This domain has been strongly implicated in trimer assembly during SR-A biosynthesis (Ashkenas *et al.,* 1993). Circular dichroism spectroscopy analyses of recombinant mutants of human SR-A have demonstrated that the seven-residue sequence Ile^{173}–Ser^{179} is essential for receptor oligomerization (Frank *et al.,* 2000). The remaining five sites for N-linked glycosylation are located in this domain. A pH-dependent conformational change within this domain is believed to be responsible for intracellular dissociation of endocytosed ligands and recycling of the receptor (Doi *et al.,* 1994).

Domain V is the collagenous domain and mediates the binding of ligands, including modified lipoproteins (Doi *et al.,* 1993). This domain contains 24 (bovine, murine, and rabbit) or 23 (human) Gly–X–Y amino acid sequences (where X or Y is any residue; in SR-A, 14 of the 24 Y residues are either proline or lysine). These residues are predicted to be neutral or positively charged at physiological pH (Kodama *et al.,* 1990; Rohrer *et al.,* 1990), with the clusters of positively charged residues highly conserved across species (Ashkenas *et al.,* 1993). Unpaired lysine residues at the outer edge of the helix would be available for intermolecular interactions with negatively charged ligands. Collagen-like motifs are also found in many secreted molecules, including mannose-binding protein and collectins and the complement component C1q (Hoppe and Reid, 1994). The latter displays binding characteristics similar to those of SR-A, with the exception that it does not recognize modified lipoprotein (Acton *et al.,* 1993). Studies of the properties of a series of truncation and point mutant constructs have revealed that the carboxyl-terminal 22 amino acids contain the site of acLDL recognition (Doi *et al.,* 1993). Mutation of Lys^{337} of the bovine protein abolished all acLDL binding. However, a wider structure–activity study of the collagenous domain of the rabbit receptor indicated that residues distinct from the terminal 22 amino acids also affect the binding of modified lipoprotein (Andersson and Freeman, 1998). The apparent

discrepancy between these data sets may be a result of the temperatures at which the binding studies were performed. In the study of Andersson and Freeman, some of the mutant receptors displayed differential activity dependent on whether the assays were performed at 37 or 4°C, suggesting conformational shifts of SR-A that influenced ligand binding. Therefore, ligand recognition apparently requires complex conformational interactions independent of the terminal collagenous sequences, which may explain the failure of short peptide models to mimic the properties of the entire domain (Mielewczyk *et al.,* 1996; Anachi *et al.,* 1995).

Domain VI defines SR-A type. The type II receptor contains a truncated C-terminal domain of 17 (human) or 6 (murine and bovine) residues. In contrast, the type I receptor contains a hinge (VIa; 7 residues) and a cysteine-rich domain (SRCR) (VIb; 101 residues) at the C terminus. At present no definitive role for this domain has been demonstrated. In one report, the SRCR domain influenced the affinity of lipopolysaccharide binding to Chinese hamster ovary (CHO)cells transfected with murine SR-A (Ashkenas *et al.,* 1993). Although the function of this domain remains obscure, as a structural motif it has been highly conserved and is found in an increasing number of different proteins, including other immune system proteins such as CD5 and CD6, across a wide range of species (Freeman *et al.,* 1990; Resnick *et al.,* 1994). A detailed comparison of all the SRCR sequences has identified at least two separate subclasses (Aruffo *et al.,* 1997). Crystallographic data of the cysteine-rich domain of Mac-2 binding protein have been reported, and this domain is believed to be a valid template for the whole SRCR superfamily (Hohenester *et al.,* 1999). CD163, a molecule that is composed of multiple SRCR domains, has been identified as a receptor that scavenges hemoglobin through the endocytosis of haptoglobin–hemoglobin complexes (Kristiansen *et al.,* 2001).

Although neither solution nuclear magnetic resolution spectroscopy nor crystallographic data are available, Resnick *et al.* (1996) have reported covalent structures of soluble SR-AI and SR-AII by electron microscopy and biophysical methods that resemble the structures predicted from the primary amino acid sequence. Rotary shadowing and negative staining of samples of purified protein revealed that they appear as extended molecules which consist of two adjacent fibrous segments: the α-helical coiled coil ($\sim$23 nm including spacer domain) and the collagenous triple helix ($\sim$21 nm). The fibrous domains are joined by a flexible hinge with a variable angle between the two domains; at its most extended form it resembles the structure predicted from the primary sequence, but at physiological pH the structure is more compact, with the collagen domain bent back on the coiled coil (Fig. 2). The functional significance of these alternative forms is unclear.

B. Gene Structure

The organization of the genes for both human and murine SR-A has been reported (Aftring and Freeman, 1995; Emi *et al.,* 1993). Both are located on chromosome 8 of the respective species. The coding regions of the gene are spread across 11 exons;

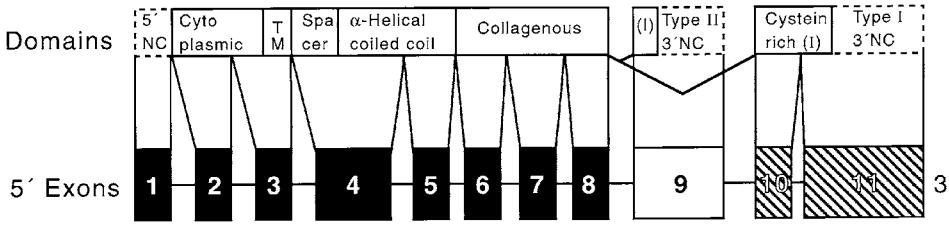


FIG. 3 Organization of the gene encoding human scavenger receptor class A. Exons are numbered, with the encoded protein domain indicated at the top (reproduced with permission from Emi *et al.,* 1993).

the first 8 encode the sequences that are common to both types of receptor (Fig. 3). Type I is generated through splicing of exon 8 to exons 10 and 11, which encode the SRCR domain. Type II results from splicing of exon 8 to exon 9, which includes the C-terminal structure. Gough *et al.* (1998) reported a third splice variant, type III, that arises from the splicing of exon 8 directly onto exon 11, which results in a novel shorter cysteine-rich region of 44 amino acids. When this form of SR-A was transfected into CHO cells, it was not expressed at the cell surface and was unable to internalize acLDL but could inhibit the binding of lipoprotein to SR-AI and SR-AII by acting as a dominant negative receptor. It is present at low levels in primary human Mϕ in culture but at higher levels in Mϕ of rabbits fed a high-fat diet. An interesting proposition is that this splice form could act as a regulatory mechanism, but currently there is no *in vivo* evidence.

The upstream regulatory regions of both human and murine genes have been studied in detail (Moulton *et al.,* 1994; Aftring and Freeman, 1995). Although comparisons of the results of promoter–activity studies have revealed differences between the two species, in general there is a reasonable consensus regarding the fragments that are necessary for specifying expression. A 291-bp fragment of the human proximal promoter in combination with a 400-bp upstream enhancer element, which contain binding sites for the transcription factors PU.1, AP-1, and ets, is sufficient to direct specific expression to Mϕ and atherosclerotic lesions in transgenic mice (Moulton *et al.,* 1994). The composite AP-1/ets motifs, which act as oxidant responsive elements, may explain phorbol ester stimulation of SR-A expression (Wu *et al.,* 1994). Recently, a 180-kb clone from a bacterial artificial chromosome library that presumably contains all the regulatory regions of the human SR-A gene has been used to generate a transgenic mouse (de Winther *et al.,* 1999).

Two polymorphisms of the SR-A gene have been discovered, one in the mouse and one in human. The inbred mouse strain C57/B16 has four amino acid substitutions in the α-helical coiled coil domain of the receptor (Fig. 4) (Daugherty *et al.,* 2000; Fortin *et al.,* 2000). A single amino acid substitution ($Lys^{168}Ser$) affects immunoreactivity with a specific mAb, but none of the differences have been shown to perturb biological functions. Second, a human family has been identified in

```
DBA/2        MTKEMTENQRLCPHEREDADCSSESVKFDARSMTASLPHSTKNGPSVQEKL
C57/B6       MTKEMTENQRLCPHEQEDADCSSESVKFDARSMTASLPHSTKNGPSLQEKL
                        <                    Cytoplasmic domain
DBA/2        KSFKAALIALYLLVFAVLIPVVGIVTAQLLNWEMKNCLVCSRNTSDTSQGP
C57/B6       KSFKAALIALYLLVFAVLIPVVGIVTAQLLNWEMKNCLVCSLNTSDTSQGP
                           ><     Transmembrane      ><     Spacer region
DBA/2        MEKENTSNVEMRFTIIMAHMKDMEERIQSISNSKADLIDTGRFQNFSMATD
C57/B6       MEKENTSKVEMRFTIIMEHMKDMEERIESISNSKADLIDTERFQNFSMATD
                                 ><
DBA/2        QRLNDILLQLNSLILSVQEHGNSLDAISKSLQSLNMTLLDVQLHTETLHVR
C57/B6       QRLNDILLQLNSLISSVQEHGNSLDAISKSLQSLNMTLLDVQLHTETLNVR


DBA/2        VRESTAKQQEDISKLEERVYKVSAEVQSVKEEQAHVEQEVKQEVRVLNNIT
C57/B6       VRESTAKQQEDISKLEERVYKVSAEVQSVKEEQAHVEQEVKQEVRVLNNIT
                              Alpha-helical coiled-coil
DBA/2        NDLRLKDWEHSQTLKNITFIQGPPGPQGEKGDRGLTGQTGPPGAPGIRGIP
C57/B6       NDLRLKDWEHSQTLKNITFIQGPPGPQGEKGDRGLTGQTGPPGAPGIRGIP
                                       ><
DBA/2        GVKGDRGQIGFPGGRGNPGAPGKPGRSGSPGPKGQKGEKGSVGGSTPLKTV
C57/B6       GVKGDRGQIGFPGGRGNPGAPGKPGRSGSPGPKGQKGEKGSVGGSTPLKTV
                             Collagenous domain              ><
DBA/2        RLVGGSGAHEGRVEIFHQGQWGTICDDRWDIRAGQVVCRSLGYQEVLAVHK
C57/B6       RLVGGSGAHEGRVEIFHQGQWGTICDDRWDIRAGQVVCRSLGYQEVLAVHK
                                    Cysteine-rich domain
DBA/2        RAHFGQGTGPIWLNEVMCFGRESSIENCKINQWGVLSCSHSEDAGVTCTS
C57/B6       RAHFGQGTGPIWLNEVMCFGRESSIENCKINQWGVLSCSHSEDAGVTCTS
                                                               >
```

FIG. 4 Comparison of the amino acid sequences of type I class A scavenger receptor from the mouse strains DBA/2 and C57/B6. Single amino acid polymorphisms are underlined.

which some members display extensive xanthelasmas and planar xanthomas and have a four- to sevenfold increase in expression of SR-A (Giry *et al.,* 1996).

C. Conservation and Evolution of SR-A

Cloning of the cDNAs encoding SR-A from four mammalian species revealed remarkable conservation of the protein, with approximately 70% homology between the amino acid sequences. The percentage sequence identity for each of the domains of the receptor is as follows: domain I, 70%; domain II, 79%; domain II, 59%; domain IVa, 61%; domain IVb, 78%; domain V, 73%; domain VIa (type I), 23%; domain VIb (type I), 81%; and domain VI (type II), 22%. However, there is evidence of species differences in properties of the receptor. Although the Re form of lipopolysaccharide (LPS) could inhibit high-affinity binding of acLDL to murine type I receptor, it had no effect on binding of acLDL by the bovine receptor (Ashkenas *et al.,* 1993). This may be due to differences in sequence of certain Gly–X–Y triplets in the collagenous domain. We have a much more limited understanding of the evolutionary origin and relationships of SR-A. Although SR activities have been identified in relatively simple animals such as *Drosophila* (Abrams *et al.,* 1992; Pearson, 1996), no molecules that display obvious sequence homologies to SR-A have been isolated. Because we currently only have knowledge of mammalian SR-A genes, it may be more appropriate to search for related molecules in other vertebrate phyla rather than in invertebrates. Expressed sequence tags which have homology to mammalian SR-A have been deposited in the database from the sequencing projects of the zebrafish and *Xenopus* genomes.

D. Expression of SR-A

The availability of specific reagents to murine (Hughes *et al.,* 1995), bovine (Naito *et al.,* 1991), and human (Matsumoto *et al.,* 1990) receptors has enabled the distribution of SR-A to be determined in many species. With the exception of polyclonal antisera raised against peptides specific for either human SR-AI or SR-AII (Naito *et al.,* 1992), we have less information on the expression patterns of the two receptor types. Coexpression of type I and type II receptors was seen in atherosclerotic streaks, Kupffer cells, and alveolar Mϕ, but recently the specificity of these antipeptide antibodies has been questioned (Honda *et al.,* 1998).

Figure 5; see color insert shows immunohistochemical staining of a variety of healthy murine tissues with the mAb 2F8. This rat antibody was isolated by virtue of its property to inhibit divalent cation-independent adhesion of the murine Mϕ cell line RAW 264 to tissue culture plastic surfaces in the presence of serum (Fraser *et al.,* 1993). This reagent reacts with both receptor types I and II. Staining is predominantly Mϕ specific, but it is restricted to subpopulations in the various

organs (Hughes *et al.,* 1995). It is prominent in Mϕ in the liver, lung, red pulp and marginal zone of the spleen, cortex and medulla of the thymus, heart, testis, lymph node, and lamina propria of the gut. There is some evidence of cellular expression that is species-dependent; in the mouse liver, SR-A is present on both Kupffer cells and the sinusoidal endothelium (Fig. 5), whereas in human it is restricted to the former (Matsumoto *et al.,* 1990). In the murine system, expression is essentially confined to the myeloid lineage, whereas immunoreactivity of smooth muscle cells and fibroblasts has been recorded in rabbit (Bickel and Freeman, 1992; Li *et al.,* 1995). In human, kidney mesangial cells (Ruan *et al.,* 1997) and high endothelial of peripheral lymph nodes (Geng and Hansson, 1995) have been reported to express SR-A antigen *in situ*. Together, these studies provide evidence consistent with SR-A contributing to biological functions, independent of pathological situations. The association of SR-A with disease, namely atherogenesis, has been shown in multiple investigations that have demonstrated its presence on the Mϕ foam cells characteristic of lipid accumulation in human lesions (Naito *et al.,* 1992) and murine and rabbit models (de Winther *et al.,* 2000).

1. Monocyte–Macrophage Differentiation and Mouse Embryogenesis

Mϕs derive from monocytes born in the bone marrow that circulate and enter tissues in which they differentiate into their mature phenotype. Although it has been known for some time that freshly isolated monocytes can endocytose modified lipoproteins that are ligands for SR-A, it has been contentious whether they actually express SR-A or if uptake of ligand occurs via a distinct SR. In order to resolve this question, peripheral blood monocytes (PBMCs) were prepared from both wild-type (strain 129) mice and mice genetically deficient in SR-A, which constitute the ideal negative control (Suzuki *et al.,* 1997), and analyzed for receptor expression and endocytosis by flow cytometry. Figure 6 shows that PBMCs from SR-A$^{-/-}$ animals did not stain with the 2F8 mAb and they endocytosed significant amounts of 1′-dioctadecyl—3,3,3′3′ tetramethylindo-carbocyanine perchlorate (diI)-labeled acLDL, which was not inhibited with the antibody against SR-A but was partially blocked with the less specific ligand polyguanylic acid. This latter observation suggests that SRs on murine monocytes other than SR-A contribute to the endocytosis of lipoproteins. It is perhaps pertinent to the possible identity of this activity that human PBMCs express the class B receptor CD36 (Huh *et al.,* 1996).

The embryonic expression pattern of SR-A has not been fully characterized, but analyses reported to date indicate that, as in the adult, it is restricted to the myeloid lineage. In their examination of phagocyte differentiation during mouse embryogenesis, Lichanska *et al.* (1999) found that SR-A transcripts were detectable at Day 11.5 dpc in a subset of cells positive for the colony-stimulating factor receptor c-*fms*. Cells were labeled in the heart, liver, and limb bud but were conspicuously less frequent than cells that expressed another Mϕ endocytic receptor,

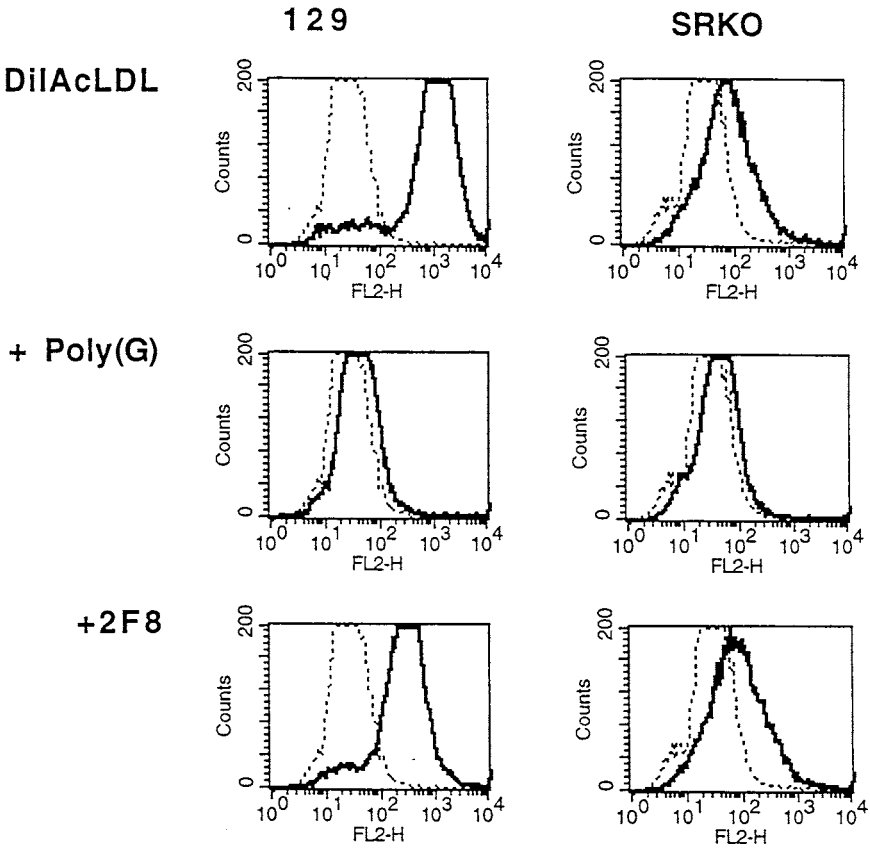


FIG. 6 Endocytosis of DiI-acLDL by murine monocytes derived from 129 and SR-A$^{-/-}$ mice. FACS profiles of monocytes exposed to fluorescent modified lipoprotein alone or in the presence of either polyguanylic acid or antiscavenger receptor class A monoclonal antibody 2F8.

the macrophage mannose receptor. Interestingly, these workers commented that SR-A positive cells showed a morphology consistent with being actively involved in phagocytosis of dying cells, which has also been observed by the authors (N. Platt and S. Gordon, unpublished observations). It may be that involvement in the process of the clearance of apoptotic cells is the major role for SR-A during embryogenesis.

2. Regulation of the Expression of SR-A

Because the central theme of this review is that SR-A is a multifunctional Mϕ molecule, it is perhaps not surprising that there have been many reports on the regulation of receptor expression in a range of biological and pathological situations.

However, it is difficult to define broad principles. Different populations of cells (which have included monocytes, Mϕ, endothelial, fibroblasts, and smooth muscle) have been examined using various experimental approaches (e.g., the measurement of biological activity, specific mRNA, or protein and examination *in vitro* or *in vivo*); therefore, comparison is not straightforward. Results of some of these studies may appear contradictory; for example, dexamethasone treatment has been reported to both upregulate (Hirsch and Mazzone, 1986) and downregulate (Moulton *et al.,* 1992) the receptor. We do not attempt a comprehensive review. Instead, we describe the regulatory mechanisms as they relate to particular biological processes and the consequences of dysregulation for disease progression.

3. Cytokine and Growth Factor Regulation of SR-A Expression

The expression levels of SR-A have been shown to be influenced by many secreted molecules, including both cytokines and growth factors, which are characteristic of normal and pathologic microenvironments. Some of these factors promote and others inhibit receptor activity, and because these molecules may coexist at specific sites and times, it is probable that the level of SR-A may be continuously regulated. Obviously, of particular interest have been the effects of those secreted factors that are known to be present locally in developing atherosclerotic lesions and plaques, with the view of determining whether they might enhance or impair SR-A expression and hence atherogenesis. However, most studies have not discriminated between effects on undefined SR activity and those specific to SR-A.

Macrophage colony-stimulating factor (M-CSF) is an important growth stimulator for the Mϕ lineage (Witte, 1990). Treatment of biogel-elicited peritoneal Mϕ with the cytokine enhances SR-A expression, which is reflected in enhanced endocytosis and adhesion (de Villiers *et al.,* 1994). However, M-CSF is not required for SR-A expression because positive immunoreactivity is detectable on some of the Mϕ populations that remain in osteopetrotic mice (N. Platt and S. Gordon, unpublished observations), which lack the cytokine (Wiktor-Jedrzejczak *et al.,* 1990).

In contrast, several-cytokines, including interferon-gamma (IFN-γ) (Geng and Hansson, 1992), transforming growth factor-beta 1 (TGF-β) (Bottalico *et al.,* 1991), tumor necrosis factor-alpha (TNF-α) (Hsu *et al.,* 1996), and interleukin-6 (IL-6) (Liao *et al.,* 1999), diminish SR-A activity *in vitro*. Treatment of human monocyte-derived Mϕ and THP-1 cells with IL-6 resulted in impaired transcription, probably because of a reduction in the binding of specific factors to elements in the promoter in the gene (Liao *et al.,* 1999). In the case of TNF-α, decreased lipoprotein binding was primarily caused by a significant reduction in SR-A message half-life (Hsu *et al.,* 1996). Therefore, it appears that these cytokines do not downregulate SR-A through a shared mechanism.

Specific growth factors may also promote SR-A expression on non-Mϕ cells, at least *in vitro*. When applied together, platelet-derived growth factor, TGF-β, epidermal growth factor, and insulin-like growth factor stimulated SR-A gene

expression and receptor activity on rabbit and human smooth muscle cells (Gong and Pitas, 1995). This suggests that SR-A-mediated uptake of modified LDL by smooth muscle cells may also lead to lipid accumulation and foam cell formation *in vivo* as proposed in Mϕ.

III. Ligand-Binding Properties of SR-A

The feature of SR-A that almost certainly underlies its contributions to a multitude of biological processes is its broad, high-affinity ligand-binding property (Krieger and Herz, 1994; Platt *et al.,* 1998). This feature is shared with members of other classes of SRs and sets SR-A apart from the majority of membrane receptors that can bind only a single ligand. Hence, SRs have been termed "molecular flypaper" (Krieger *et al.,* 1993). Ligands (Table I) comprise a diverse array of molecules,

TABLE I
Ligands for SR-A[a]

Ligands	Nonligands
Acetylated low-density lipoprotein (acLDL) Oxidized low-density lipoprotein (oxLDL) Malondialdehyde LDL Glycoaldehyde LDL	Low-density lipoprotein (LDL) High-density lipoprotein (HDL) Methylated LDL
Maleylated bovine serum albumin (mal-BSA) Formaldehyde-treated albumin	Bovine serum albumin (BSA) Acetylated BSA
Fucoidan Dextran sulfate Carragheenan	Heparin Chondroitan sulfate Mannan
Polyguanylic acid Polyinosinic acid	Polycytidylic acid Polyadenylic acid
	Phosphatidylserine Phosphatidylcholine
Glycated collagen IV Advanced glycation end products	Native collagen
Crocidolite asbestos Silica Polyvinyl sulfate	
Lipopolysaccharide Lipotechoic acid	
β-Amyloid	

[a]Receptor recognition or nonrecognition has been shown for these molecules either through direct binding studies or through the inhibition of modified lipoprotein endocytosis.

including nonphysiological ones (such as acLDL), molecules derived from the environment (such as silica particles and crocidolite asbestos), or ones that are microbial in origin (such as LPS of gram-negative bacteria). The identification of molecules as ligands for SR-A can be informative and predictive about potential roles for the receptor.

Chemical modifications that include acetylation and oxidation convert LDL into a ligand for SR-A and abolish binding to the LDL receptor. A minimum modification of about 15–20% of the lysine residues of LDL is sufficient to facilitate binding to SR-A (Zhang *et al.,* 1993). A second interesting and important feature of the binding properties of SR-A is the phenomenon of "nonreciprocal cross-competition." That is, although the binding of ligand A to the receptor can be completely competed out with an excess of ligand B, the reverse is not true. An example involves the binding of acLDL and oxidized LDL (oxLDL). Competition of the former for SR-A by the latter is absolute, but when the situation is reversed, inhibition is only partial (Fig. 7) (Freeman *et al.,* 1991; Ashkenas *et al.,* 1993).

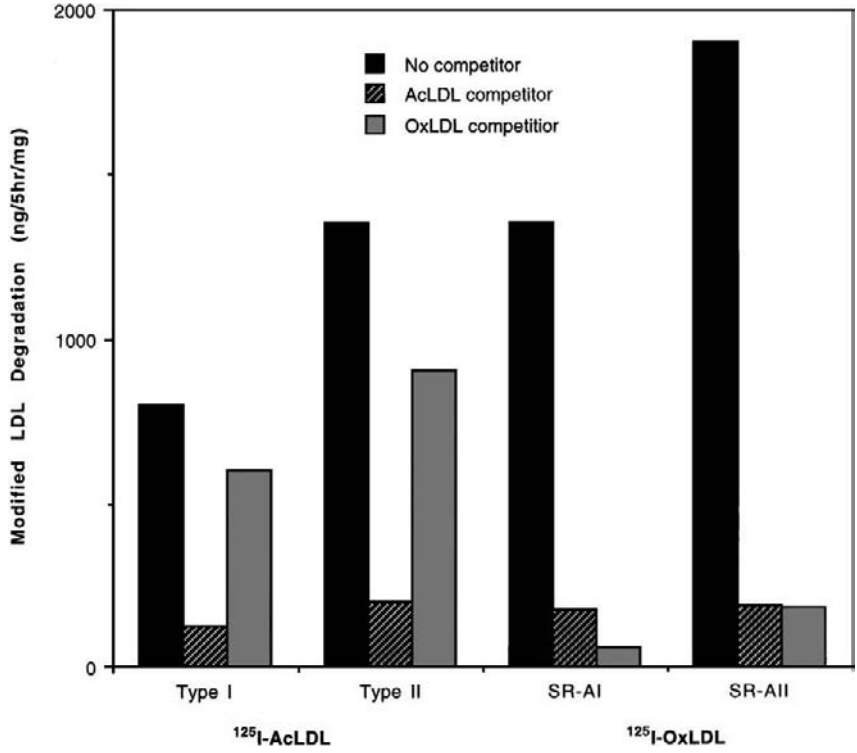


FIG. 7 Nonreciprocal ligand cross-competition of transfected CHO cells expressing type I or type II scavenger receptor class A. Cross-competition of unlabeled acLDL and oxLDL for ^{125}I-acLDL and ^{125}I-oxLDL degradation (redrawn with permission from Freeman *et al.,* 1991).

This implies the existence of distinct but probably overlapping binding sites on SR-A for the two modified lipoproteins. This adds a further degree of complexity to potential interactions.

Although the list of ligands is long, it is apparent that it is incomplete. Biological situations in which SR-A is believed to function are known, but there is little information on the identity of the specific ligand. For example, the serum component necessary for SR-A-mediated adhesion of Mϕ to tissue culture plastic is still unidentified (Fraser *et al.,* 1993). The characterization of endogenous ligands is poor, particularly for those that mediate cell-to-cell interactions mediated by SR-A, and the very broad array of those polyanionic molecules that have been documented as ligands makes it difficult to predict the nature of new ones. Even when ligands have been identified *in vitro,* it is not certain that SR-A is responsible for their binding *in vivo*. SR-A has been shown to bind and internalize unopsonized environmental particles such as oxide particles and quartz (Kobzik, 1995); alveolar Mϕ, which would be the route of entry of these materials into the body, express high levels of SR-A. However, alveolar Mϕ from SR-A$^{-/-}$ mice showed no decrease in particle binding compared with wild-type cells. Rather, a mAb directed against the other class A SR, MARCO, showed immunoreactivity on alveolar Mϕ and was able to partially inhibit particle binding (Palecanda *et al.,* 1999). Therefore, these tissue Mϕ express two different species of SR, both of which are capable of recognizing these pollutant particles, but only one of them appears to function *in vivo*. There are many possible explanations for this, including differential affinities of the receptors for these ligands.

Hughes *et al.* (1995) investigated the identity of tissues rich in endogenous ligands for SR-A. In order to examine SR-A-mediated Mϕ adhesion in a context relevant to the interactions that occur within lymphoid and nonlymphoid organs, they employed an assay of Mϕ adhesion to frozen tissue sections (i.e., a Stamper–Woodruff assay). The chelation of magnesium and calcium ions revealed specific patterns of adhesion of the murine Mϕ cells RAW 264.7 to tissues that were inhibited with the specific mAb 2F8. Among those tissue regions to which strong adherence was observed were the subcapsular and medullary regions of the lymph node, the lamina propria of the gut, areas around the major airways of the lung, the red pulp and marginal zone of the spleen, and broad areas of the liver. That this adherence occurred in the absence of exogenous fetal bovine serum, which is a requirement for SR-A-dependent adhesion to tissue culture plastic, confirms that these tissues contain alternative ligands for SR-A.

Although the precise structural requirement for binding to SR-A have not been fully determined, all ligands are macromolecular and polyanionic, but the latter property alone is insufficient to confer recognition by the receptor. One system that has been investigated to ascertain the structural requirements for ligand binding is SR-A recognition of polyribonucleotides (Pearson *et al.,* 1993). Polyguanylic acid (poly (G)) and polyinosinic acid (poly(I)) can bind to SR-A, whereas the related molecules polycytidylic acid and polyadenylic acid cannot. Originally this

was considered to be true because only the former could form a specific quadruplex structure, but a reexamination of the interaction suggested that binding occurs because poly(G) and poly(I) can form aggregates (Suzuki *et al.,* 1999). This observation may implicate SR-A in the uptake and removal of endogenous complexes, such as those composed of nucleic acids or immune complexes.

IV. Biological Activities of SR-A

A. General

1. Endocytosis of Modified Lipoproteins and Atherogenesis

As previously mentioned, SRs are defined by their ability to endocytose modified lipoprotein and hence best known for their contribution to lipoprotein metabolism. SRs are not downregulated by high levels of intracellular cholesterol, which results in lipid accumulation and the conversion of Mϕ into foam cells characteristic of atherogenesis. Because of the health implication of this pathological process, it remains the major focus of research efforts. This area of research is outside the scope of this article but has been the subject of many detailed reviews (de Winther *et al.,* 2000; Glass and Witztum, 2001).

The one aspect of atherogenesis that we consider demonstrates the importance of the generation of mice genetically deficient in types I and II SR-A. Prior to this, the contribution of a specific SR to lipoprotein endocytosis was difficult to define *in vivo* because of the presence of a multitude of different receptors with apparently overlapping activities. This also applies to other activities of SR-A that we consider subsequently.

SR-A null mice were produced by targeted disruption of exon 4 of the gene that is essential for formation of functional receptors (Doi *et al.,* 1994). Receptor-deficient mice were viable and fertile (Suzuki *et al.,* 1997). *In vitro* uptake of acLDL by elicited peritoneal Mϕ from these animals was found to be only about 20% of that of wild-type cells (Fig. 8). The contribution of SR-A to the development of atherosclerosis was examined by crossing SR-A$^{-/-}$ animals with mice lacking apolipoprotein E, which display a high frequency of spontaneous vascular disease (Suzuki *et al.,* 1997). The double-mutant animals displayed a reduction of about 60% of lesions. This showed the importance of SR-A to the development of atherosclerosis, but the residual activity confirmed the participation of other SRs. The latter was confirmed by the subsequent observations that the clearance of injected oxLDL is unaffected in mice that lack SR-A (Ling *et al.,* 1997; Lougheed *et al.,* 1997; Van Berkel *et al.,* 1998) and that mice lacking the class B scavenger receptor CD36 also show less lesion development (Febbraio *et al.,* 2000). This theme of receptor redundancy or the presence of compensatory mechanisms applies to some of the other situations in which SR-A is believed to function. Mice

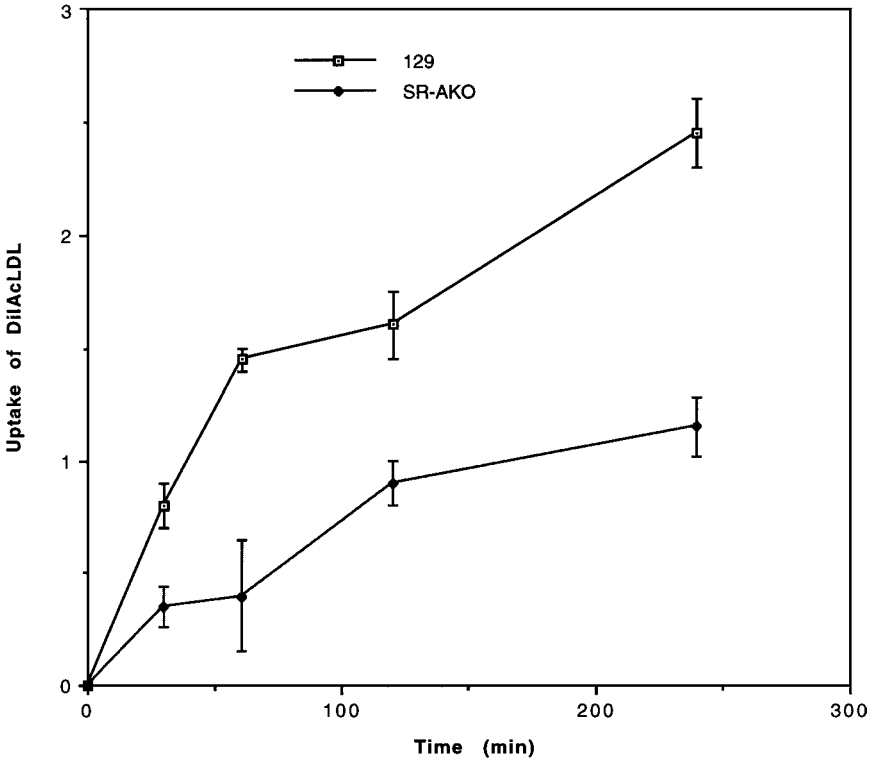


FIG. 8 Endocytosis of acLDL by elicited peritoneal Mϕ from 129 and SR-A$^{-/-}$ mice.

lacking both SR-A and low-density lipoprotein receptor showed less reduction in high-fat diet-induced lesions (Sakaguchi *et al.,* 1998). However, the apparently damaging role of SR-A has been challenged by the occurrence of diminished lesions in transgenic mice overexpressing the human gene (de Winter *et al.,* 1999). A recent study indicated that variation in genetic backgrounds has a strong and perhaps critical influence on the effect of receptor modulation in different mouse models (Mazzone, 2000).

2. Macrophage Adhesion

The ability of Mϕ to migrate and be retained in tissues and at sites of infection and inflammation is vital. Attachment also signals changes in the biological properties of the adhered cell. Mϕ express a selection of adhesion receptors that bring them into intimate contact with other cells or the extracellular matrix (Hogg and Landis, 1993). An adhesive capacity for SR-A was shown when a mAb (2F8) that could block cation-independent adhesion of Mϕ to tissue culture plastic in the presence

of serum was discovered to be directed against SR-A (Fraser *et al.,* 1993). This activity for the receptor was confirmed with the observations that peritoneal Mϕ derived from SR-A$^{-/-}$ mice display delayed adherence and spreading *in vitro* (Suzuki *et al.,* 1997) (Fig. 9) and that transfection of the gene into weakly adherent cells can confer an adhesive phenotype (Robbins and Horlick, 1998). The adhesion to tissue culture plastic surfaces is due to an unidentified ligand(s) in serum. The presence of alternate ligands in various lymphoid and nonlymphoid organs (which include specific regions of the spleen, thymus, lung, and gut) was shown by the demonstration of SR-A-mediated adhesion under serum-free conditions (Hughes *et al.,* 1995). The nature of this ligand(s) has not been identified.

The adhesive properties of SR-A may indicate that the receptor promotes atherosclerosis not solely through its endocytic activity but also by facilitating Mϕ retention during lesion development. Consistent with this hypothesis, van Velzen *et al.* (1999) found that SR-A displays the adhesion property *in vitro* only when Mϕ have been activated, which would mirror their condition in the inflammatory microenvironment of the vessel wall. Whether the failure of SR-A to contribute to the adhesion of unstimulated resident cells is a result of insufficient surface expression, ineffective conformation, or the absence of critical components of SR-A adhesion complexes remains to be determined. This study did reveal an *in vivo* adhesion deficit of the SR-A null mouse; the number of Kupffer cells that could be isolated from the liver was reduced, suggesting that SR-A may play an important role in either adhesion or differentiation.

SR-A has been shown to adhere Mϕ to ligands associated with specific disease conditions. Adhesion to both glucose-modified collagen IV (El Khoury *et al.,* 1994) and advanced glycation end products (Araki *et al.,* 1995), which may underlie the accelerated development of vascular problems in diabetics, and also to oxLDL-coated surfaces (Maxeiner *et al.,* 1998) occurs via SR-A. This apparently induces specific secretory responses of the Mϕ, which are believed to initiate a sequence of inflammatory events. A possible role for SR-A in the induction of the pathology of Alzheimer's diseases has been suggested by data showing that SR-A can adhere microglia to the amyloid fibrils present in the plaques that are characteristic of the disease (Christie *et al.,* 1996; Honda *et al.,* 1998). This adhesive event stimulates production of potentially damaging reactive oxygen species and cytokines (El Khoury *et al.,* 1996). SR-A can also mediate endocytosis of aggregates of amyloid β protein (Paresce *et al.,* 1996). Correlating with these experiments, expression of SR-A has been shown on microglia of brains containing Alzheimer lesions (in healthy tissue it is restricted to perivascular cells), but it is not clear if SR-A is primarily involved in promoting the disease or expressed secondarily as a result of damage. In fact, transgenic mice overexpressing human amyloid protein precursors showed no inhibition or delay in plaque formation when crossed onto the SR-A-deficient mouse strain (Huang *et al.,* 1999). It is known that SR-A expression can be induced by different injuries to the central nervous system (Bell *et al.,* 1994).

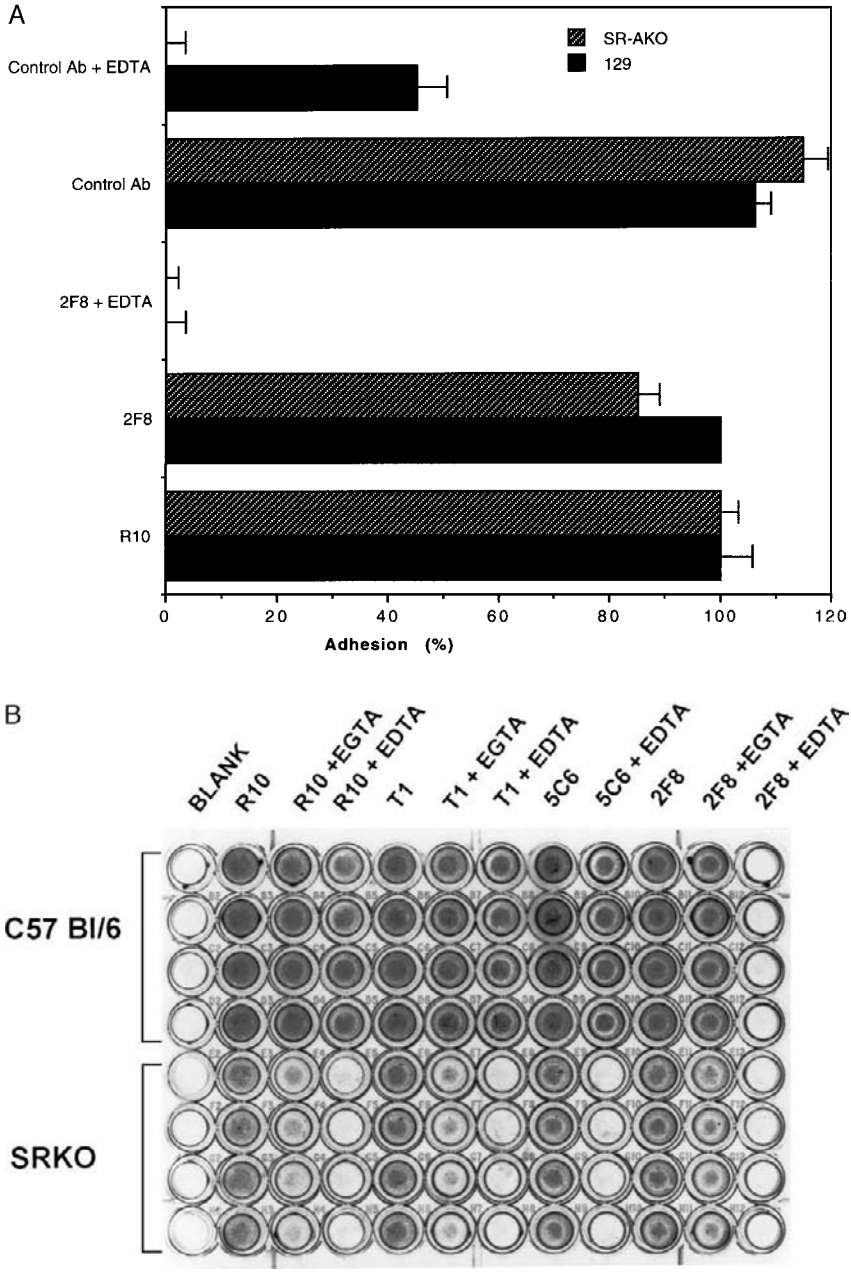


FIG. 9 (A) Adhesion of 129 and SR-A$^{-/-}$ peritoneal macrophages to fetal calf serum-coated tissue culture plastic. Cells were plated in a 96-well plate in RPMI/10% fetal calf serum alone or in the presence of anti-class A macrophage scavenger receptor monoclonal antibody (2F8) or an isotype-matched control in the absence of divalent cations. (B) Photographic image of adhesion of C57b/6 and SR-A$^{-/-}$ peritoneal Mϕ to a 96-well plate in the presence of serum plus the presence or absence of anti-SR-A mAb (2F8) and anti-complement receptor 3 mAb (5C6) and divalent cations.

SR-A may promote adhesion of Mϕ directly to other cells to facilitate cell–cell interactions. Hansson and co-workers (Yokota *et al.,* 1998) have shown that certain activated B cell lines can adhere to SR-A transfected CHO cells by a mechanism that does not require the polyanion binding site of the receptor. This observation, coupled with the reports of SR-A expression by human high endothelial cells of postcapillary venules and follicular dendritic cells (Geng and Hansson, 1995), suggests a role in trafficking and recruitment of B lymphoblasts that could be tested in the SR-A$^{-/-}$ mouse.

B. Roles in Innate Immunity

1. SR-A as a Pattern Recognition Receptor

The observation that SR-A expression is specific to Mϕ strongly implies a contribution to innate immunity. Within the adaptive response of the host to infectious challenge, there are several stages at which contributions by SR-A can be anticipated. An important component of the defense strategy of the innate system is the possession of receptors that are capable of discriminating structures that are restricted in their distribution to microbes (or "non-self"). These have been collectively termed pattern recognition molecules or receptors (PRRs) by Janeway (1992) and include complement, collectins, mannose receptors, and perhaps members of the Toll family of receptors, which are part of the signaling pathway of innate immunity (Aderem and Ulevitch, 2000). All of these are able to recognize particular molecules shared among but restricted to pathogens, such as LPS of gram-negative bacteria, glycolipids of mycobacteria, and lipoglycans and lipoteichoic acids of gram-positive bacteria (Medzhitov and Janeway, 2000; Ulevitch and Tobias, 1999). The recognition of these structures by an appropriate receptor(s) on the phagocyte can bring about microbe binding, phagocytosis, and induction of antimicrobial activities.

Critical to the argument that SR-A might behave as a PRR were the demonstrations that LPS of gram-negative and lipoteichoic acid (LTA) of gram-positive bacteria both bind to the receptor (Hampton *et al.,* 1991; Dunne *et al.,* 1994). The uptake of lipid IV_A, the active moiety of endotoxin, by Mϕ was blocked by many of the known ligands of SR-A, and its binding to CHO cells transfected with bovine type I or type II receptors was inhibited with ligands of the same specificity (Ashkenas *et al.,* 1993). Similarly, LTA was shown to bind to soluble type I SR-A and could compete with ligands such as poly(G) in a binding assay (Dunne *et al.,* 1994). Greenberg *et al.* (1996) subsequently investigated a series of LTA and lipoglycan species derived from different bacterial strains for their ability to bind. Gram-positive bacteria can be roughly divided into two subgroups—those that have LTAs as the major macroamphiphile of the membrane and those that have replaced it with various lipoglycans. Therefore, evaluation of the ability of

these molecules to bind would not only allow an understanding of the structural requirements for recognition but also implicate those microbes with which SR-A may interact. It was found that both the size and the distribution of the negative charge of the side chain phosphate groups of LTA determined the capacity and strength of binding. Specific lipoglycans were reported to bind to an equivalent extent. Potentially significant was the finding that the lipoglycan of *Micrococcus luteus* did not bind, which may cast doubt on a report that blockade of SRs can inhibit invasion of human Mϕ by *Mycobacterium tuberculosis* because it has a structurally similar lipoglycan (Zimmerli *et al.,* 1996).

Exposure of Mϕ to LPS has been shown to influence the expression of SR-A in a species-dependent manner. In human monocyte-derived Mϕ, LPS exposure decreases SR activity and downregulates SR-A message levels (Van Lenten *et al.,* 1985; Van Lenten and Fogelman, 1992). Paradoxically, in the murine system, the opposite appears to occur (Fitzgerald *et al.,* 2000): Endotoxin induced SR-A mRNA through a posttranscriptional mechanism that promoted either message maturation or export. Interestingly, TNF-α treatment of mouse cells could not mimic the effects of LPS, indicating that the increase was not the result of autocrine/paracrine production of the cytokine, which is in contrast to human responses, in which the downregulation of SR-A is mediated via TNF-α (Van Lenten and Fogelman, 1992). A possible explanation is that the principal LPS receptors in the two species differ in their ability to effect production of the cytokine, which may explain the relative resistance of mice toward endotoxemia. Second, the regulatory regions of the human and mouse genes may differ in the presence of response elements for endotoxin-induced transcription factors.

Systemic delivery of LPS into mice resulted in increased expression on splenic Mϕ of red and white pulp and on the sinusoidal endothelium and Kupffer cells of the liver within 6 h. The increase was maximal at 24 h and declined to normal levels by 2 days (Fitzgerald *et al.,* 2000). In a separate study, direct injection of endotoxin into the mouse hippocampus also caused significant upregulation of constitutive receptor expression on stromal, epiplexus, and meningeal Mϕ and induced expression on resident microglia in the region by 24 h (Bell *et al.,* 1994). Upregulation may result from increased receptor recycling, but the latter observation suggests that expression of SR-A can result from signals transduced as a result of endotoxin binding to another receptor(s). This revealed SR-A to be a marker of microglial activation and suggested potential roles in either damping down an ongoing response or resolving the local tissue damage that may result from cell activation.

2. Metabolism and Response to LPS

One of the potentially severe consequences of failure to control gram-negative infection is the development of sepsis and endotoxic shock as a result of the release of LPS (Redl *et al.,* 1993). This molecule activates host cells, which induces an uncontrolled proinflammatory response that can cause multiple organ failure

and even death. Studies of myeloid cells have identified many receptors that are involved in the binding of LPS that result in activation (Ulevitch and Tobias, 1995). CD14, in both membrane-bound and soluble forms, and LPS-binding protein can mediate binding and uptake that initiate a signaling cascade that culminates in the production of proinflammatory and effector cytokines, such as TNF-α and IL-6 (Wright, 1995). A recently identified family of receptors, termed Toll receptors because they are evolutionarily related to the *Drosophila* gene *Toll,* have also been shown to respond to various pathogen-associated molecules, including endotoxin, and to induce signals that activate the adaptive immune response (Aderem and Ulevitch, 2000). The roles of each of these have been investigated by examining the responses to LPS of mice genetically engineered or naturally lacking specific receptors. Involvement in cell activation has been demonstrated for CD14 (Ulevitch and Tobias, 1995), TLR2, and TLR4 because of the hyporesponsiveness of the respective null mice (Aderem and Ulevitch, 2000).

Circulating LPS is known to be rapidly cleared by the liver (Ulevitch and Tobias, 1995) and Kupffer and sinusoidal endothelium display high SR activity (Hughes *et al.,* 1995) and express SR-A (Fig. 5). Ligands for SR-A (and other SRs), coinjected with LPS, significantly reduced hepatic uptake, implying SR-A might facilitate clearance and detoxification of the lipid (Hampton *et al.,* 1991). Haworth *et al.* (1997) examined this hypothesis by comparing the responses of live *Bacillus Calmette Guerin* (BCG)-infected SR-A$^{-/-}$ and SR-A$^{+/+}$ mice to endotoxic shock. Null mice were found to be more susceptible and produced greater quantities of TNF-α and IL-6 in response to LPS, confirming that SR-A protects the host by both scavenging endotoxin and limiting the release of proinflammatory cytokines. It also seems likely, though not formally shown, that internalization via SR-A does not lead to cellular activation. The increased sensitivity of SR-A$^{-/-}$ mice compared with the increased resistance of CD14$^{-/-}$ mice supports the hypothesis that the response of an individual cell is a reflection of the amount of endotoxin that is internalized via activating receptor(s) (e.g., CD14) and nonactivating ones (e.g., SR-A). Figure 10 depicts the model, which shows examples of each of the two populations of receptors. The diversity of receptors for LPS that have been recognized and the potential for additional, as yet uncharacterized, others obviously add greater complexity. Additionally, if the signaling pathways engaged by receptors are not distinct but intersect, there could be integration of signal intensities that could further refine the overall cellular outcome. The molecular interactions that are stimulated by the binding of endotoxin to CD14 and TLRs continue to be a subject of intense study. Unfortunately, our knowledge of the downstream events that follow SR-A engagement is limited.

3. SR-A and the Phagocytosis of Microbes

Many Mϕ plasma membrane receptors have been shown to contribute to opsonic and nonopsonic recognition and uptake of bacteria (Mosser, 1994). Platt *et al.*

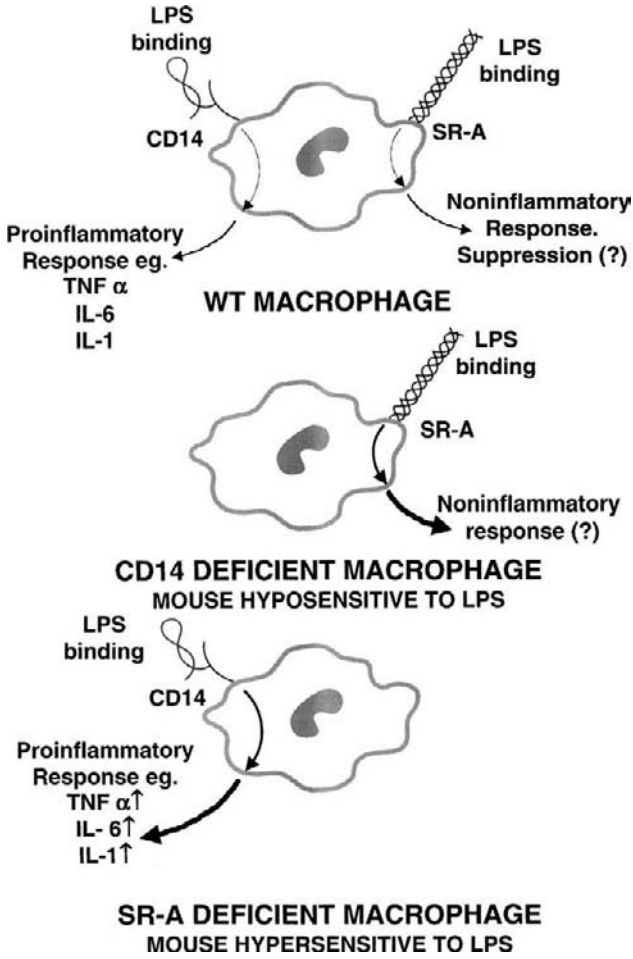


FIG. 10 Possible mechanism for the response of wild-type, CD14$^{-/-}$, and SR-A$^{-/-}$ macrophages to lipopolysaccharide.

(1999) exploited the availability of the knockout mouse to show that there is an *in vitro* reduction in uptake of heat-killed *Escherichia coli* of about 30% by activated SR-A$^{-/-}$ peritoneal Mϕ. In the original paper that described the first study of the receptor-deficient mice, the mice were reported to be more susceptible to infection with *Listeria monocytogenes* (Suzuki *et al.,* 1997). SR-A$^{-/-}$ Kupffer cells displayed impaired phagocytosis and killing of the bacterium compared to wild-type cells, and hepatic granulomas were more extensive in the deficient mice (Ishiguro *et al.,* 2001). A greater sensitivity to infection with herpes simplex virus was also briefly mentioned, but more detailed information has not been published.

Peiser *et al.* (2000) have undertaken an alternative strategy in an attempt to determine the specific contribution of SR-A by examining its contribution in the absence of other receptors that recognize bacteria. CHO cells stably expressing human SR-A bound *E. coli* and *Staphylococcus aureus,* whereas untransfected cells did not bind but few microbes were ingested. This implies that SR-A cannot mediate efficient internalization in this particular cell type or that receptor density on the cell or that of the ligand on the microbe is insufficient. Greater binding was observed for cells bearing type II SR-A than for cells bearing the type I isoform, which contains the cysteine-rich domain. As might be predicted, bone-marrow-derived Mϕ prepared from SR-A$^{-/-}$ mice ingested fewer bacteria than wild-type phagocytes. The extent of bacterial uptake was influenced by the strain of *E. coli* used: Ingestion of the *K1* strain was reduced to a greater extent than that of DH5α, which suggests differential distribution of ligand(s). Interestingly, capsulated bacteria such as *E. coli K1,* in which LPS may be masked, also bound, suggesting the presence of alternate ligands.

The nature of the interaction between SR-A and the gram-positive pathogen *S. aureus* has been further studied by Thomas *et al.* (2000). They compared the progression of infection in wild-type and SR-A$^{-/-}$ mice inoculated intraperitoneally with the microbe. Clearance of the bacterium from the peritoneum and blood was decreased in SR-A$^{-/-}$ animals: In wild-type mice less than 0.3% of the original inoculum was recovered from the peritoneum after 24 h and no bacteria were present in the blood at 12 h. In knockout mice that received an identical dose, 20% of the initial load was still present in the peritoneum at 24 h and bacterial counts in the blood had increased 200-fold. The impaired ability to eliminate *S. aureus* resulted in diminished survival of SR-A$^{-/-}$ mice. Wild-type animals were able to control bacteria doses up to 2×10^8 CFU, at which only 40% of deficient animals survived. When the challenge was increased to 1×10^9 CFU, all SR-A$^{-/-}$ animals became moribund within 12 h. This data set of comparative susceptibility of the two strains of mice to gram-positive infection closely resembles that observed when they were exposed to LPS challenge and confirms that SR-A functions in the innate immune response to both gram-negative and gram-positive infections.

4. Granuloma Formation

In the mouse, systemic spread of pathogens such as BCG mycobacteria to the spleen and liver results in the cellular recruitment of cells to the foci of infection. The granuloma that is formed is a mechanism employed by the host to attempt to limit the spread of the pathogen. The cytokine milieu of the microenvironment activates the recruited Mϕ, which results in increased microbicidal and cytocidal activities (Gordon *et al.,* 1994). Haworth *et al.* (1997) found no appreciable difference in the numbers, adhesion, and endocytic activities of activated Mϕ recruited to BCG

granulomata in wild-type and SR-A null mice. Therefore, in contrast to IFN-γ-deficient mice, SR-A$^{-/-}$ mice were not more susceptible to this bacterium and were able to control infection. Although Hagiwara and colleagues (1999) found no increase in susceptibility of the knockout mice, they reported delayed Mϕ recruitment during the early states (first 10 days) of hepatic granuloma formation in response to intravenous injection of *Corynebacterium parvum*. The normal pattern of cell numbers and size of granulomas were subsequently established by 14 days. Impairment in the production of monocyte chemoattractant protein-1, TNF-α, and IFN-γ was also recorded in the initial 3 days postinfection. Therefore, SR-A is involved in granuloma development in response to *C. parvum,* but the ability of the host to resolve the infection is not compromised significantly in its absence. It is possible that alternate receptor(s) mechanisms were activated to compensate for SR-A. The idea that the extent of the contribution of SR-A may be dependent on the nature of the microbe is an attractive hypothesis. *Listeria monocytogenes,* to which it has been reported SR-A$^{-/-}$ mice are more susceptible (Suzuki *et al.,* 1997), also causes granulomatous–type infection (Ishiguro *et al.,* 2001). This model therefore deserves investigation to ascertain why the outcome differs significantly from the two systems just described.

5. Phagocytosis of Apoptotic Cells

The efficient phagocytosis of cells that die by apoptosis or programmed cell death is an important process (Platt *et al.,* 1998; Savill, 1997). The rapid uptake by professional phagocytes or neighbors ensures removal of dying cells before the possibility of lysis and unwelcome exposure to their intracellular contents. Most studies of apoptotic cell clearance have concentrated on identification of the receptor(s) on the plasma membrane of the phagocyte that mediates recognition. Interestingly, although no single receptor has been identified as responsible for all ingestion, many that have been implicated are members of the different classes of SRs (Platt and Gordon, 1998). Indeed, this may represent the ancestral function of this family of receptors. The phagocytosis of apoptotic cells shares with bacterial binding the theme of pattern recognition—the cell surfaces of both differ from that of the healthy eukaryotic cell. If the microbe can be considered "infectious nonself," then the apoptotic cells represents "altered self"; therefore, perhaps it is not surprising that common recognition systems are involved. However, a vitally important difference between the two is the consequence of ingestion: Phagocytosis of foreign cells usually leads to the generation of a proinflammatory response, whereas apoptotic cell uptake is nonstimulatory and may even suppress a concurrent inflammatory stimulus (Fadok *et al.,* 1998).

There is evidence of SR-A as a receptor for the recognition and internalization of apoptotic thymocytes. Thymic Mϕ expressed the receptor both *in situ* and when isolated from the organ; in an *in vitro* phagocytosis assay, the mAb blocked

approximately 50% of ingestion, and an equivalent reduction in phagocytosis was seen when Mϕ prepared from SR-A$^{-/-}$ thymi were tested (Platt *et al.,* 1996). The extent of inhibition was comparable to that reported for other candidate phagocytic receptors (Platt *et al.,* 1998)—it is increasingly apparent that binding and ingestion are complex interactions that require the activity of more than a single receptor. Analyses of apoptotic thymocyte clearance *in vivo* failed to reveal a significant phenotype consistent with impaired clearance (i.e., an accumulation of apoptotic corpses) (Platt *et al.,* 2000). Although alternative explanations for the unaltered phagocytosis of the null mouse are possible, the most likely explanation is receptor redundancy. Activated platelets are cells that are technically not apoptotic (according to the definition of apoptotic death being executed through caspase(s) enzymatic activity), but behave in immunological terms very much like apoptotic cells (i.e., they are identified as being unwanted by phagocytes and cleared). These aged or dying cells were shown to be ingested *in vitro* by Mϕ via SR-A (Brown *et al.,* 2000). Therefore, not only may SR-A be important in the phagocytosis of cells undergoing different forms of programmed cell death but also initiation of the caspase cascade is not essential for the generation of its phagocytic ligands.

We have found that SR-A can fulfill the criteria required to define it as a phagocytic receptor (that it can transfer phagocytic activity when expressed in a nonphagocytic cell). CHO cells transfected with SR-A were able to ingest particles bearing appropriate ligands and apoptotic neutrophils (Platt *et al.,* 1998). Therefore, SR-A mediates ingestion and not purely binding, and it may be involved in the removal of apoptotic granulocytes at sites of inflammation. One component of the system for which we currently have little information is the nature of the ligand(s) on the apoptotic cell specific for SR-A. The most characterized apoptotic ligand, phosphatidylserine, which is translocated to the outer leaflet of the plasma membrane early in the apoptotic program (Martin *et al.,* 1995), is not recognized by SR-A (Lee *et al.,* 1992). Perhaps relevant to identification of a ligand is the observation of Terpstra *et al.* (1997), who found that unlike the binding of apoptotic thymocytes, that of oxidized erythrocytes to SR-A$^{-/-}$ Mϕ was not diminished. This suggests that the ligand(s) generated during apoptosis is not a result of this chemistry.

C. Roles in Adaptive Immune Response

One of the more intriguing revelations that has emerged from the renewed interest in the innate immune system is the demonstration that components have been conserved and adopted as stimulators of adaptive immunity (Medzhitov and Janeway, 2000). For example, studies of various members of the Toll family have shown that LPS binding elicits the production of cytokines and upregulation of

costimulatory molecules (Aderem and Ulevitch, 2000). Although the available data do not suggest that ligation of SR-A by endotoxin has a similar effect, studies have been indicative of other potential roles in the adaptive response. It appears that the pattern recognition property of SRs may be exploited for the endocytosis and presentation of suitably modified antigens (Abraham *et al.,* 1995). This may be analogous to the CD1 system of the presentation of glycolipids (Porcelli and Modlin, 1999).

Rath and colleagues demonstrated that the targeting of proteins to SRs, through chemical modifications such as maleylation, leads to enhanced immunogenicity and antigenicity (Abraham *et al.,* 1995, 1997; Singh *et al.,* 1998). Soluble diphtheria toxin was very poor at eliciting a significant antibody response, but when the soluble protein was maleylated (mDT), a considerable response resulted. In addition, immunization with the modified toxin gave a better T cell proliferation, even in the absence of adjuvant. A large proportion of the B cell epitopes recognize the maleylation structure, suggesting that maleyl-lysine is the hapten. In contrast, the T cell epitopes generated seemed to be primarily directed against the native protein: T cell responses in animals immunized with mDT could be recalled with the native molecule and vice versa. The enhanced responsiveness may be due to the higher levels of peptide–MHC complex formed with mDT antigen and their increased persistence (Singh *et al.,* 1998). The modifications that convert proteins to SR ligands also shift the immune response toward more of a type 1 cytokine profile, with enhanced IFN-γ production and less IL-4 and IL-10. Bansal *et al.* (1999) reported that maleylated proteins could undergo presentation by both MHC class I and class II restriction. However, the ligands and animals used in these studies did not permit discrimination between the specific contributions of SR-A and those of other SRs. Nicoletti *et al.* (1999) have begun to address this problem through a comparison of wild-type and SR-A$^{-/-}$ cells. They immunized with oxLDL and maleylated murine serum albumin and found that strong proliferative responses were produced in the wild-type but not in the SR-A$^{-/-}$ mice. Addition of SR-A^{+} antigen presenting cells to splenic cultures from oxLDL-injected SR-A$^{-/-}$ animals restored T cell responses, indicating that SR-A can mediate uptake of antigen for presentation to antigen-specific T cells. However, they found that SR-A knock out mice were still able to mount an antigen-dependent T cell response *in vivo,* suggesting that another receptor could mediate antigen uptake and presentation. Together, these reports provide a basis for further studies of the cell biology of SR-A, its role in antigen presentation, and the cellular fate of endocytosed and phagocytosed antigens. A particularly attractive model is one that utilizes an endogenous, physiologically relevant ligand for SR-A. A recent dramatic finding is that antigen derived from apoptotic cells can enter the class I pathway of dendritic cells (Albert *et al.,* 1998). Because SR-A has been strongly implicated in the phagocytosis of apoptotic cells, it is of obvious interest to determine whether SR-A is involved in this aspect.

V. Cell Biology of SR-A

A. SR-A Engagement, Signal Transduction, and Macrophage Function

Previously, we summarized the evidence that SR-A can mediate internalization of soluble and particulate ligands through endocytosis and phagocytosis. These two mechanisms differ in many ways; for example, the former is typically a clathrin-based mechanism, whereas phagocytosis requires action polymerization. However, they share many common features, including the trafficking of membrane and the delivery of the internalized material into discrete intracellular compartments that undergo sequential maturation. It is clearly of genuine interest to appreciate the molecular interactions that enable SR-A to internalize through two distinct pathways.

SR-A spontaneously gathers in coated pits on the cell surface even in the absence of ligand binding. Amino acid sequences within the cytoplasmic domains that are needed for receptor association with components of clathrin-coated pits, and hence enable internalization, have been identified for several endocytic receptors. For example, YXRF of the transferrin receptor (Collawn *et al.,* 1990) and NPXY of the LDL receptor (Chen *et al.,* 1990) are required for internalization. All the motifs identified to date have been predicted to form a tight turn structure. The cytoplasmic tail of SR-A apparently does not have any of the internalization sequences found in these other molecules, but a secondary structure prediction algorithm suggested two possible sites encoding turn sequences (Asp^{13}–Glu^{19} and Asn^{38}–Thr^{41}) of the bovine receptor. In order to investigate the structural basis of internalization, Morimoto and colleagues (1999) undertook a deletion mutagenesis study of the N-terminal region of the cytoplasmic tail and the two candidate motifs. They examined the efficiency of internalization of bound acLDL when each of the mutant receptors was expressed in COS-7 cells. Sequential removal of amino acids suggested that the region between amino acids Thr^{18} and Ser^{27} included the sequence critical for efficient endocytosis. Alanine scanning mutagenesis of this sequence demonstrated that mutations of Val^{21}, Phe^{23}, Asp^{24}, and Val^{28} not only greatly reduced internalization of ligand but also reduced cell surface expression of the receptor. Therefore, the motif VXFD is essential for coated pit-mediated endocytosis and receptor trafficking. Importantly, these residues lie outside the two motifs the authors predicted to form possible turn structures; rather, it is the sequence Val^{21}–Val^{28} that is responsible for highly effective endocytosis. Secondary structure modeling of this motif strongly implies that it adopts a helical structure, which has also been predicted for the internalization motif of CD4 and others. Attempts were made to confirm this configuration through circular dichroism studies of a synthetic 18-amino-acid peptide model of the domain but were unsuccessful (it adopted a random coiled structure). This may reflect a simple deficiency in

length of the peptide or a more complicated inability to form the trimeric structure required for secondary conformation. However, counter to this analysis of bovine SR-A, Fong and Le (1999) made a similar study of the murine receptor and reported overtly similar results. Based on the application of a different secondary structure prediction algorithm, they proposed the existence of four motifs, two of which overlap with those of Morimoto *et al.* (1999). Only the alanine replacement of Asp^{25} (which is the terminal amino acid of the bovine VXFD motif) inhibited acLDL degradation. Mutagenesis of the other candidate motifs did not influence internalization, but because inhibition with Ala^{25} was only 50%, the involvement of other cytoplasmic regions needs to be examined further.

The cytoplasmic domains encoded by cDNAs isolated from the four species include three conserved serine/threonine phosphorylation sites (Ashkenas *et al.,* 1993). Mouse peritoneal Mϕ were cultured with protein phosphatase and protein kinase inhibitors to investigate whether phosphorylation of these residues influenced receptor activity (Fong, 1996). The phosphatase inhibitor okadaic acid inhibited acLDL binding by approximately 70%, despite normal levels of plasma membrane expression, suggesting that this was due to either SR-A inactivation or sequestration of receptors to sites which made them inaccessible to ligands. On the other hand, the kinase inhibitor staurosporin increased receptor expression at the cell surface by 30% but slowed the kinetics of ligand internalization. These results suggested that phosphorylation/dephosphorylation of SR-A may be an important regulatory mechanism, but the study could not demonstrate a clear correlation between receptor activity and the degree of phosphorylation of the cytoplasmic tail. Site-directed mutagenesis of murine SR-A of the potential phosphorylation sites showed detectable phosphorylation only of the serine residues Ser^{21} and Ser^{49} and not of Thr^{30} (Fong and Le, 1999). Interestingly, the requirement for phosphorylation of each of the serine residues is distinct. Substitution of Ser^{21} increased acLDL metabolism because of an increase in cell surface expression of the receptor. On the other hand, inactivation of Ser^{49} reduced lipoprotein uptake through a slowing of the kinetics of receptor internalization. Normalization of endocytic activity was seen in cells that expressed receptor mutant for both phosphorylation sites, confirming that the two sites do not act independently and that SR-A processing of the ligand therefore requires a specific pattern of phosphorylation. Because elimination of Ser^{49} did not completely block receptor internalization, it seems, that the recognition site for coated pit targeting is not this amino acid but lies in a separate region of the cytoplasmic domain. The available data implicate the motif surrounding Asp^{25} and this could be tested by examining the properties of the double-mutant Ser^{49}/Asp^{25}. One other intriguing result from this study was that the kinetics of phosphorylation of SR-A is also ligand dependent. oxLDL stimulated a lower maximal increase in phosphorylation than did acLDL, and it declined to basal levels more rapidly. This may explain the mechanism by which these two lipoprotein ligands stimulate distinguishable responses from the Mϕ. This is likely the result of differential activation of SR-A because acLDL and

oxLDL bind at separate, although probably overlapping, sites in the extracellular collagenous domain.

Although SR-A-mediated endocytosis of modified lipoproteins is considered a major component of the mechanism that transforms Mϕ into foam cells, it seems probable that receptor engagement may also signal other events that accelerate the atherosclerotic process. The current understanding of the diseased vasculature is that it resembles an inflammatory microenvironment in that it is characterized by the presence of an array of growth factors, proinflammatory molecules, cytokines, chemoattractants, and invasion by inflammatory cells (Ross, 1999). It seems likely that these changes result either directly or indirectly from the signaling events that arise from lipoprotein–receptor interactions.

Many studies have investigated the influence of SR-A ligands on Mϕ, particularly in relation to lesion development. The effects may also be pertinent to influencing Mϕ activities in other situations. oxLDL has been shown to be mitogenic for both Mϕ and vascular smooth muscle cells *in vitro* and this may be the mechanism through which the number of Mϕ in the arterial intima increases during the early stages of lesion development (Glass and Witztum, 2001). However, because Mϕ in lesions are known to express many receptors which can bind oxLDL *in vitro,* such as CD36, SR-B1, Lox-1, and macrosialin/CD68, it cannot be concluded that the growth stimulatory effect is due to SR-A-mediated lipoprotein uptake. This multitude of receptors makes interpretation difficult of all studies in which cells have been exposed to ligands that do not discriminate between different classes of scavenger receptor. For the sake of argument, if it is accepted that the induction of Mϕ growth by oxLDL is primarily the result of SR-A-mediated endocytosis, evidence indicates that it requires an increase in phosphatidylinositol (PI) 3-kinase activity. The specific inhibitors wortmannin and LY294002 reduced the mitogenic effect of oxLDL by about 50% (Martens *et al.,* 1998). One interesting observation that lends support to the ideas of either the involvement of receptors other than SR-A in stimulating Mϕ growth or that all ligands do not interact with the receptor identically is that acLDL cannot mimic the mitogenic effects of oxLDL (Sakai *et al.,* 1994). We mentioned previously that these two lipoproteins display nonreciprocal cross-competition for SR-A and their distinguishable effects on Mϕ proliferation may be due to differential conformational changes of the receptor upon ligation and subsequent generation of distinctive signals.

An alternative explanation may be that SR-A mediates uptake of lysophosphatidylcholine, a component of oxLDL which is the active moiety (Martens *et al.,* 1998). However, oxLDL and acLDL have both been shown to elicit many other signaling responses, including the activation of MAP kinase (Deigner and Claus, 1996) and stimulation of specific protein kinase C isoforms (Claus *et al.,* 1996). The latter is in accordance with the location of two consensus sites for protein kinase C phosphorylation in the cytoplasmic tail of the receptor (Ashkenas *et al.,* 1993). There may also be an association between SR-A and the tyrosine kinase

Lyn (Miki *et al.,* 1996). The two molecular forms of the kinase, p53/p56, are tyrosine phosphorylated within 30 s of the exposure of THP-1 cells to acLDL and *lyn* could be coimmunoprecipitated with SR-A from cell lysates. Interestingly, *lyn*-deficient mice develop only very reduced atherosclerotic lesions despite hypercholersterolemia when fed a high-fat diet (Horikawa *et al.,* 2001). However, ligands that have overlapping but not necessarily identical receptor affinities, such as oxLDL and acLDL, have been shown to stimulate disparate results. For example, although treatment of elicited murine peritoneal Mϕ with either oxLDL or maleylated bovine serum albumin produced transient increases in intracellular calcium ions and the hydrolysis of phosphatidylinositol 4,5-biphosphate, the response to oxLDL was pertussis toxin sensitive, but that induced by the modified albumin was insensitive (Misra *et al.,* 1996; Shackelford *et al.,* 1995). In a strategy to identify signal transduction pathways that are specifically triggered through SR-A (and perhaps to attempt to rationalize the sometimes conflicting reports of the responses to particular ligands), Hsu *et al.* (1998) compared the Mϕ–like cell THP-1 with a Bowes melanoma line stably transfected with SR-A. acLDL and fucoidan triggered protein tyrosine phosphorylation of many proteins, including phospholipase C-γ1 and PI-3 kinase, in both cell types. Interestingly, the kinetics of phosphorylation stimulated by the two ligands were not equivalent; that triggered by exposure to the polysaccharide returned to basal levels quickly, whereas that induced by the lipoprotein increased over time. Although the significance of this observation is unclear, it may have physiological relevance. Confirmation that SR-A can act as a signaling receptor should facilitate more critical analyses of the intracellular cascades stimulated by the binding of a range of ligands and the biological context in which it occurs, whether through endocytosis, phagocytosis, or an adhesive interaction.

The investigation of Hsu and coworkers (1998) also demonstrated that SR-A-mediated signaling could induce changes in specific gene expression. oxLDL is known to suppress the expression of specific genes induced by inflammatory stimuli (Hamilton *et al.,* 1995). Previous studies have shown that the classical nondiscriminatory ligands, such as acLDL, fucoidan, poly(I), and dextran sulfate, can upregulate the expression of urokinase-type plasminogen activator in a protein kinase C-dependent manner (Falcone *et al.,* 1991). This enhanced expression leads to plasmin-dependent extracellular matrix degradation and the release of matrix-bound growth factors. Such a scenario is considered likely to happen in the atherosclerotic lesion *in situ*. In order to test directly that the effects were dependent on SR-A-mediated signaling, Falcone *et al.* examined melanoma cells transfected with the receptor and found an identical pattern of tyrosine phosphorylation in response to binding of several of the same ligands. Therefore, there is increasing evidence that SR-A behaves as a signaling molecule, but our current understanding of the post-receptor-binding events is sketchy and we have little detailed information on the precise sequence of the signal transduction. We wish

to know how this compares in the response to the binding of different ligands, how the signals that derive from internalization via the separate endocytic and phagocytic routes compare, and the effects on Mϕ physiology.

B. Cell Growth and Differentiation

We have discussed the evidence that at least some SR-A ligands are able to stimulate Mϕ growth and may be responsible for the significant proliferation that is seen in pathological situations. However, the requirement for SR-A for Mϕ growth is not obvious and the physiological relevance is unclear. All the major populations of tissue Mϕ are apparently present in the SR-A-deficient mouse and express their normal pattern of antigens (N. Platt, R. Haworth, and S. Gordon, unpublished observations). However, growth of Mato's cells, which are fluorescent granular perithelial cells (FGPs) situated between the basal lamina of cerebral microvessels and glial limitans of neural tissue (Mato *et al.,* 1996) which express the receptor, is impaired in the SR-A$^{-/-}$ mouse (Mato *et al.,* 1997). Immunohistochemistry and electron microscopy revealed that most SR-A$^{-/-}$ FGPs fail to extend properly and retain an immature phenotype. These cells typically mature over a period of 14 days after birth, but SR-A$^{-/-}$ FGPs remain in a fibroblastoid form beyond this age and some cells totally degenerate within 4 weeks. This defect may therefore arise from the absence of a signal that is transduced from either SR-A-dependent endocytosis or adhesion. It is not clear if this requirement of SR-A for cell survival and normal maturation is specific to this lineage or is a more widespread but restricted phenomenon.

VI. Concluding Remarks

It is clear that SR-A contributes to many of the functions that Mϕ display in their capacity to regulate homeostasis, host defense, and immune function. In evolutionary terms, it appears to be a very sensible strategy to exploit those molecules that have already appeared rather than necessitate the production of new ones as different situations evolve. The unusual ligand-binding properties of SR-A accentuate the opportunities of its reuse. However, it could be that rather than the involvement of SR-A in an increasing range of biological processes, it is the reappearance of similar ligands that has driven the process. Whichever of these is true, it highlights the importance of modified proteins, lipoproteins, altered epitopes, etc. and their molecular recognition in a multitude of situations. Therefore, the identification of more endogenous ligands and analyses of their biological relevance remain priorities. It will also be of interest to define those activities among SRs that are unique to SR-A and the biological situations in which they cooperate in an additive

or synergistic fashion. The spate of recent advances in our understanding of the complex biology of SR-A has resulted from the availability of important reagents that facilitate its study. Their exploitation promises even further insights.

References

Abraham, R., Choudhury, A., Basu, S. K., Bal, V., and Rath, S. (1997). Disruption of T cell tolerance by directing a self antigen to macrophage-specific scavenger receptors. *J. Immunol.* **158,** 4029–4035.

Abraham, R., Singh, N., Mukhopadhyay, A., Basu, S. K., Bal, V., and Rath, S. (1995). Modulation of immunogenicity and antigenicity of proteins by maleylation to target scavenger receptors on macrophages. *J. Immunol.* **154,** 1–8.

Abrams, J. M., Lux, A., Steller, H., and Krieger, M. (1992). Macrophages in Drosophila embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA* **89,** 10375–10379.

Acton, S., Resnick, D., Freeman, M., Ekkel, Y., Ashkenas, J., and Krieger, M. (1993). The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands. *J. Biol. Chem.* **268,** 3530–3537.

Aderem, A., and Ulevitch, R. J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature* **406,** 782–787.

Aftring, R. P., and Freeman, M. W. (1995). Structure of the murine macrophage scavenger receptor gene and evaluation of sequences that regulate expression in the macrophage cell line, P388D. *J. Lipid Res.* **36,** 1305–1314.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998). Dendritic cells acquire antigens from apoptotic cells and induce class I-restricted CTLs. *Nature* **392,** 86–89.

Anachi, R. B., Siegel, D. L., Baum, J., and Brodsky, B. (1995). Acid destabilization of a triple-helical peptide model of the macrophage scavenger receptor. *FEBS Lett.* **368,** 551–555.

Andersson, L., and Freeman, M. W. (1998). Functional changes in scavenger receptor binding conformation are induced by charge mutants spanning the entire collagen domain. *J. Biol. Chem.* **273,** 19592–19601.

Araki, N., Higashi, T., Mori, T., Shibayama, R., Kawabe, Y., Kodama, T., Takahashi, K., Shichiri, M., and Horiuchi, S. (1995). Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction. *Eur. J. Biochem.* **230,** 408–415.

Aruffo, A., Bowen, M. A., Patel, D. D., Haynes, B. F., Starling, G. C., Gebe, J. A., and Bajorath, J. (1997). CD6–ligand interactions: A paradigm for SRCR domain functions? *Immunol. Today* **10,** 498–504.

Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1993). Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J. Lipid Res.* **34,** 983–1000.

Bansal, P., Mukherjee, P., Basu, S. K., George, A., Bal, V., and Rath, S. (1999). MHC class I-restricted presentation of maleylated protein binding to scavenger receptors. *J. Immunol.* **162,** 4430–4437.

Bell, M. D., Lopez Gonzalez, R., Lawson, L., Hughes, D., Fraser, I., Gordon, S., and Perry, V. H. (1994). Upregulation of the macrophage scavenger receptor in response to different forms of injury in the CNS. *J. Neurocytol.* **23,** 605–613.

Bickel, P. E., and Freeman, M. W. (1992). Rabbit aortic smooth muscle cells express inducible macrophage scavenger receptor messenger RNA that is absent from endothelial cells. *J. Clin. Invest.* **90,** 1450–1457.

Bottalico, L. A., Wager, R. E., Agellon, L. B., Assoian, R. K., and Tabas, I. (1991). Transforming growth factor-beta 1 inhibits scavenger receptor activity in THP-1 human macrophages. *J. Biol. Chem.* **266,** 22866–22871.

Brown, M. S., and Goldstein, J. L. (1983). Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52,** 223–261.

Brown, S. B., Clarke, M. C., Magowan, L., Sanderson, H., and Savill, J. (2000). Constitutive death of platelets leading to scavenger receptor-mediated phagocytosis: A caspase-independent cell clearance program. *J. Biol. Chem.* **275,** 5987–5996.

Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990). NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalisation of low density lipoprotein. *J. Biol. Chem.* **265,** 3116–3123.

Christie, R. H., Freeman, M., and Hyman, B. T. (1996). Expression of the macrophage scavenger receptor, a multifunctional lipoprotein receptor, in microglia associated with senile plaques in Alzheimer's disease. *Am. J. Pathol.* **148,** 399–403.

Claus, R., Fyrnys, B., Deigner, H. P., and Wolf, G. (1996). Oxidised low-density lipoprotein stimulates protein kinase C (PKC) and induces expression of PKC-isotypes via prostaglandin-H-synthase in P388D1 macrophage-like cells. *Biochemistry* **35,** 4911–4922.

Collawn, J. F., Stangel, M., Kuhn, L. A., Esekogwu, V., Jing, S., Trowbridge, I. S., and Tainer, J. A. (1990). Transferrin receptor internalisation sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell* **63,** 1061–1072.

Daugherty, A., Whitman, S. C., Block, A. E., and Rateri, D. L. (2000). Polymorphism of class A scavenger receptors in C57BL/6 mice. *J. Lipid Res.* **41,** 1568–1577.

Deigner, H. P., and Claus, R. (1996). Stimulation of mitogen activated protein kinase by LDL and oxLDL in human U-937 macrophage-like cells. *FEBS Lett.* **385,** 149–153.

de Villiers, W. J., Fraser, I. P., Hughes, D. A., Doyle, A. G., and Gordon, S. (1994). Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function. *J. Exp. Med.* **180,** 705–709.

de Winther, M. P., van Dijk, K. W., van Vlijmen, B. J., Gijbels, M. J., Heus, J. J., Wijers, E. R., van den Bos, A. C., Breuer, M., Frants, R. R., Havekes, L. M., and Hofker, M. H. (1999). Macrophage specific overexpression of the human macrophage scavenger receptor in transgenic mice, using a 180-kb yeast artificial chromosome, leads to enhanced foam cell formation of isolated peritoneal macrophages. *Atherosclerosis* **147,** 339–347.

de Winther, M. P., van Dijk, K. W., Havekes, L. M., and Hofker, M. H. (2000). Macrophage scavenger receptor class A: A multifunctional receptor in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **20,** 290–297.

Doi, T., Higashino, K., Kurihara, Y., Wada, Y., Miyazaki, T., Nakamura, H., Uesugi, S., Imanishi, T., Kawabe, Y., Itakura, H., *et al.,* (1993). Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors. *J. Biol. Chem.* **268,** 2126–2133.

Doi, T., Kurasawa, M., Higashino, K., Imanishi, T., Mori, T., Naito, M., Takahashi, K., Kawabe, Y., Wada, Y., Matsumoto, A., *et al.,* (1994). The histidine interruption of an alpha-helical coiled allosterically mediates a pM-dependent ligand dissociation from macrophage scavenger receptors. *J. Biol. Chem.* **269,** 25598–25604.

Dunne, D. W., Resnick, D., Greenberg, J., Krieger, M., and Joiner, K. A. (1994). The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc. Natl. Acad. Sci. USA* **91,** 1863–1867.

El Khoury, J., Thomas, C. A., Loike, J. D., Hickman, S. E., Cao, L., and Silverstein, S. C. (1994). Macrophages adhere to glucose-modified basement membrane collagen IV via their scavenger receptors. *J. Biol. Chem.* **269,** 10197–10200.

El Khoury, J., Hickman, S. E., Thomas, C. A., Cao, L., Silverstein, S. C., and Loike, J. D. (1996). Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils [see comments]. *Nature* **382,** 716–719.

Elomaa, O., Kangas, M., Sahlberg, C., Tuukkanen, J., Sormunen, R., Liakka, A., Thesleff, I., Kraal, G., and Tryggvason, K. (1995). Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell* **80,** 603–609.

Emi, M., Asaoka, H., Matsumoto, A., Itakura, H., Kurihara, Y., Wada, Y., Kanamori, H., Yazaki, Y., Takahashi, E., Lepert, M., *et al.* (1993). Structure, organization, and chromosomal mapping of the human macrophage scavenger receptor gene. *J. Biol. Chem.* **268,** 2120–2125.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* **101,** 890–898.

Falcone, D. J., McCaffrey, T. A., and Vergilio, J. A. (1991). Stimulation of macrophage urokinase expression by polyanions is protein kinase C-dependent and requires protein and RNA synthesis. *J. Biol. Chem.* **266,** 22726–22732.

Febbraio, M., Podrez, E. A., Smith, J. D., Hajjar, D. P., Hazen, S. L., Hoff, H. F., Sharma, K., and Silverstein, R. L. (2000). Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J. Clin. Invest.* **105,** 1049–1056.

Fitzgerald, M. L., Moore, K. J., Freeman, M. W., and Reed, G. L. (2000). Lipopolysaccharide induces scavenger receptor A expression in mouse macrophages: A divergent response relative to human THP-1 monocyte/macrophages. *J. Immunol.* **164,** 2692–2700.

Fong, L. G. (1996). Modulation of macrophage scavenger receptor transport by protein phosphorylation. *J. Lipid Res.* **37,** 574–587.

Fong, L. G., and Le, D. (1999). The processing of ligands by the class A scavenger receptor is dependent on signal information located in the cytoplasmic domain. *J. Biol. Chem.* **274,** 36808–36816.

Fortin, A., Penman, M., Stevenson, M. M., Krieger, M., and Gros, P. (2000). Identification and characterisation of naturally occurring variants of the macrophage scavenger receptor (SR-A). *Mammalian Genome* **11,** 779–785.

Frank, S., Lustig, A., Schulthess, T., Engel, J., and Kammerer, R. A. (2000). A distinct seven-residue trigger sequence is indispensible for proper coiled-coil formation of the human scavenger receptor oligomerization domain. *J. Biol. Chem.* **275,** 11672–11677.

Fraser, I., Hughes, D., and Gordon, S. (1993). Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. *Nature* **364,** 343–346.

Freeman, M., Ashkenas, J., Rees, D. J., Kingsley, D. M., Copeland, N. G., Jenkins, N. A., and Krieger, M. (1990). An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning type I and type II murine macrophage scavenger receptors. *Proc. Natl. Acad. Sci. USA* **87,** 8810–8814.

Freeman, M., Ekkel, Y., Rohrer, L., Penman, M., Freedman, N. J., Chisolm, G. M., and Krieger, M. (1991). Expression of type I and type II bovine scavenger receptors in Chinese hamster ovary cells: Lipid droplet accumulation and nonreciprocal cross competition by acetylated and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA* **88,** 4931–4935.

Geng, Y. J., and Hansson, G. K. (1992). Interferon-gamma inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. *J. Clin. Invest.* **89,** 1322–1330.

Geng, Y. J., and Hansson, G. K. (1995). High endothelial cells of postcapillary venules express the scavenger receptor in human peripheral lymph nodes. *Scand. J. Immunol.* **42,** 289–296.

Giry, C., Giroux, L. M., Roy, M., Davignon, J., and Minnich, A. (1996). Characterization of inherited scavenger receptor overexpression and abnormal macrophage phenotype in a normolipidemic subject with planar xanthomas. *J. Lipid Res.* **37,** 1422–1435.

Glass, C. K., and Witztum, J. L. (2001). Atherosclerosis: The road ahead. *Cell* **104,** 503–516.

Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979). Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA* **79,** 333–337.

Gong, Q., and Pitas, R. E. (1995). Synergistic effects of growth factors on the regulation of smooth muscle cell scavenger receptor activity. *J. Biol. Chem.* **270,** 21672–21678.

Gordon, S., Keshav, S., and Stein, M. (1994). BCG-induced granuloma formation in murine tissues. *Immunobiology* **54,** 369–377.

Gough, P. J., Greaves, D. R., and Gordon, S. (1998). A naturally occurring isoform of the human macrophage scavenger receptor (SR-A) gene generated by alternative splicing blocks modified LDL uptake. *J. Lipid Res.* **39,** 531–543.

Greenberg, J. W., Fischer, W., and Joiner, K. A. (1996). Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. *Infect. Immun.* **64,** 3318–3325.

Greenwalt, D. E., Lipsky, R. H., Ockenhouse, C. F., Ikeda, H., Tandon, N. N., and Jamieson, G. A. (1992). Membrane glycoprotein CD36: A review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* **80,** 1105–1115.

Hagiwara, S. I., Takeya, M., Suzuki, H., Kodama, T., van der Laan, L. J., Kraal, G., Kitamura, N., and Takahashi, K. (1999). Role of macrophage scavenger receptors in hepatic granuloma formation in mice. *Am. J. Pathol.* **154,** 705–720.

Hamilton, T. A., Major, J. A., and Chisolm, G. M. (1995). The effects of oxidized low density lipoproteins on inducible mouse macrophage gene expression are gene and stimulus dependent. *J. Clin. Invest.* **95,** 2020–2027.

Hampton, R. Y., Golenbock, D. T., Penman, M., Krieger, M., and Raetz, C. R. (1991). Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature* **352,** 342–344.

Haworth, R., Platt, N., Keshav, S., Hughes, D., Darley, E., Suzuki, H., Kurihara, Y., Kodama, T., and Gordon, S. (1997). The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. *J. Exp. Med.* **186,** 1431–1439.

Hirsch, J. L., and Mazzone, T. (1986). Dexamethasone modulates lipoprotein metabolism in cultured human monocyte-derived macrophages: Stimulation of scavenger receptor activity. *J. Clin. Invest.* **77,** 485–490.

Hogg, N., and Landis, R. C. (1993). Adhesion molecules in cell interactions. *Curr. Opin. Immunol.* **5,** 383–390.

Hohenester, E., Sasaki, T., and Timpl, R. (1999). Crystal structure of a scavenger receptor cysteine-rich domain sheds light on an ancient superfamily. *Nat. Struct. Biol.* **6,** 228–232.

Honda, M., Akiyama, H., Yamada, Y., Kondo, H., Kawabe, Y., Takeya, M., Takahashi, K., Suzuki, H., Doi, T., Sakamoto, A., Ookawara, S., Mato, M., Gough, P. J., Greaves, D. R., Gordon, S., Kodama, T., and Matsushita, M. (1998). Immunohistochemical evidence for a macrophage scavenger receptor in Mato cells and reactive microglia of ischemia and Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **245,** 734–740.

Hoppe, H. J., and Reid, K. B. (1994). Collectins—soluble proteins containing collagenous regions and lectin domains—and their roles in innate immunity. *Protein Sci.* **3,** 1143–1158.

Horikawa, M. S., Nishizumi, H., Suemura, M., Sato, B., Yamamoto, M., Takatsu, K., Yamamoto, T., and Miki, Y. (2001). Reduction of atherosclerosis despite hypercholesterolemia in lyn-deficient mice fed a high-fat diet. *Genes Cells* **6,** 37–42.

Hsu, H. Y., Nicholson, A. C., and Hajjar, D. P. (1996). Inhibition of macrophage scavenger receptor activity by tumor necrosis factor-alpha is transcriptionally and post-transcriptionally regulated. *J. Biol. Chem.* **271,** 7767–7773.

Hsu, H. Y., Hajjar, D. P., Khan, K. M., and Falcone, D. J. (1998). Ligand binding to macrophage scavenger receptor-A induces urokinase-type plasminogen activator expression by a protein kinase-dependent signaling pathway. *J. Biol. Chem.* **273,** 1240–1246.

Huang, F., Buttini, M., Wyss Coray, T., McConlogue, L., Kodama, T., Pitas, R. E., and Mucke, L. (1999). Elimination of the class A scavenger receptor does not affect amyloid plaque formation or neurodegeneration in transgenic mice expressing human amyloid protein precursors. *Am. J. Pathol.* **155,** 1741–1747.

Hughes, D. A., Fraser, I. P., and Gordon, S. (1995). Murine macrophage scavenger receptor: In vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. *Eur. J. Immunol.* **25,** 466–473.

Huh, H. Y., Pearce, S. F., Yesner, L. M., Schindler, J. L., and Silverstein, R. L. (1996). Regulated expression of CD36 during monocyte-to-macrophage differentiation: Potential role of CD36 in foam cell formation. *Blood* **87,** 2020–2028.

Ishiguro, T., Naito, M., Yamamoto, T., Hasegawa, G., Gejyo, F., Mitsuyama, M., Suzuki, H., and Kodama, T. (2001). Role of macrophage scavenger receptors in response to *Listeria* monocytogenes infection in mice. *Am. J. Pathol.* **158,** 179–188.

Janeway, C. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today* **13,** 11–16.

Jing, S., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I. S. (1990). Role of the human transferrin receptor cytoplasmic domain in endocytosis: Localisation of a specific signal sequence for internalisation. *J. Cell Biol.* **110,** 283–294.

Kobzik, L. (1995). Lung macrophage uptake of unopsonized environmental particulates. Role of scavenger-type receptors. *J. Immunol.* **155,** 367–376.

Kodama, T., Reddy, P., Kishimoto, C., and Krieger, M. (1988). Purification and characterization of a bovine acetyl low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA* **85,** 9238–9242.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., and Krieger, M. (1990). Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature* **343,** 531–535.

Krieger, M. (1999). Charting the fate of the "good cholesterol": Identification and characterisation of the high-density lipoprotein receptor. *Annu. Rev. Biochem.* **68,** 523–558.

Krieger, M., and Herz, J. (1994). Structures and functions of multiligand lipoprotein receptors: Macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63,** 601–637.

Krieger, M., Acton, S., Ashkenas, J., Pearson, A., Penman, M., and Resnick, D. (1993). Molecular flypaper, host defense, and atherosclerosis: Structure, binding properties, and functions of macrophage scavenger receptors. *J. Biol. Chem.* **268,** 4569–4572.

Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O., Hoffman, H.-J., Law, S. K. A., and Moestrup, S. K. (2001). Identification of the haemoglobin scavenger receptor. *Nature* **409,** 198–201.

Lee, K. D., Pitas, R. E., and Papahadjopoulos, D. (1992). Evidence that the scavenger receptor is not involved in the uptake of negatively charged liposomes by cells. *Biochim. Biophys. Acta* **1111,** 1–6.

Li, H., Freeman, M. W., and Libby, P. (1995). Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokines. *J. Clin. Invest.* **95,** 122–133.

Liao, H. S., Matsumoto, A., Itakura, H., Doi, T., Honda, M., Kodama, T., and Geng, Y. J. (1999). Transcriptional inhibition by interleukin-6 of the class A macrophage scavenger receptor in macrophages derived from human peripheral monocytes and the THP-1 monocytic cell line. *Arterioscler. Thromb. Vasc. Biol.* **19,** 1872–1880.

Lichanaska, A. M., Browne, C. M., Henkel, G. W., Murphy, K. M., Ostrowski, M. C., McKercher, S. R., Maki, R. A., and Hume, D. A. (1999). Differentiation of the mononuclear phagocyte system during mouse embryogenesis: The role of the transcription factor PU.1. *Blood* **94,** 127–138.

Ling, W., Lougheed, M., Suzuki, H., Buchan, A., Kodama, T., and Steinbrecher, U. P. (1997). Oxidized or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of the scavenger receptor class A type I/II gene. *J. Clin. Invest.* **100,** 244–252.

Lougheed, M., Lum, C. M., Ling, W., Suzuki, H., Kodama, T., and Steinbrecher, U. (1997). High affinity saturable uptake of oxidized low density lipoprotein by macrophages from mice lacking the scavenger receptor class A type I/II. *J. Biol. Chem.* **272,** 12938–12944.

Martens, J. S., Reiner, N. E., Herrera-Velit, P., and Steinbrecher, U. P. (1998). Phosphatidylinositol 3-kinase is involved in the induction of macrophage growth by oxidised low density lipoprotein. *J. Biol. Chem.* **273,** 4915–4920.

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182,** 1545–1556.

Mato, M., Ookawara, S., Sakamoto, A., Aikawa, E., Ogawa, T., Mitsuhashi, U., Masuzawa, T., Suzuki, H., Honda, M., Yazaki, Y., Watanabe, E., Luoma, J., Yla Herttuala, S., Fraser, I., Gordon, S., and

Kodama, T. (1996). Involvement of specific macrophage-lineage cells surrounding arterioles in barrier and scavenger function in brain cortex. *Proc. Natl. Acad. Sci. USA* **93,** 3269–3274.

Mato, M., Ookawara, S., and Sakamoto, A. (1997). Growth retardation of Mato's fluorescent granular perithelial (FGP) cells in scavenger receptor knockout (SRKO) mice. *Anat. Rec.* **247,** 307–316.

Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori, H., Aburatani, H., Takaku, F., Suzuki, H., *et al.* (1990). Human macrophage scavenger receptors: Primary structure, expression, and localization in atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* **87,** 9133–9137.

Maxeiner, H., Husemann, J., Thomas, C. A., Loike, J. D., El Khoury, J., and Silverstein, S. C. (1998). Complementary roles for scavenger receptor A and CD36 of human monocyte-derived macrophages in adhesion to surfaces coated with oxidized low-density lipoproteins and in secretion of H_2O_2. *J. Exp. Med.* **188,** 2257–2265.

Mazzone, T. (2000). Scavenger receptors in atherosclerosis: New answers, new questions. *Arterioscler. Thromb. Vasc. Biol.* **20,** 2506–2508.

Medzhitov, R., and Janeway, C. J. (2000). Innate immune recognition: Mechanisms and pathways. *Immunol. Rev.* **173,** 89–97.

Mielewczyk, S. S., Breslauer, K. J., Anachi, R. B., and Brodsky, B. (1996). Binding studies of a triple-helical peptide model of macrophage scavenger receptor to tetraplex nucleic acids. *Biochemistry* **35,** 11396–11402.

Miki, S., Tsukada, S., Nakamura, Y., Aimoto, S., Hojo, H., Sato, B., Yamamoto, M., and Miki, Y. (1996). Functional and possible physical association of scavenger receptor with cytoplasmic tyrosine likase Lyn in monocytic THP-1-derived macrophages. *FEBS Lett.* **399,** 241–244.

Misra, U. K., Shackelford, R. E., Florine Casteel, K., Thai, S. F., Alford, P. B., Pizzo, S. V., and Adams, D. O. (1996). Maleylated-BSA induces hydrolysis of PIP2, fluxes of Ca^{2+}, NF-kappaB binding, and transcription of the TNF-alpha gene in murine macrophages. *J. Leukocyte Biol.* **60,** 784–792.

Morimoto, K., Wada, Y., Hinagata, J., Imanishi, T., Kodama, T., and Doi, T. (1999). VXFD in the cytoplasmic domain of macrophage scavenger receptors mediates their efficient internalization and cell-surface expression. *Biol. Pharm. Bull.* **22,** 1022–1266.

Mosser, D. M. (1994). Receptors on phagocytic cells involved in microbial recognition. *Immunol. Ser.* **60,** 99–114.

Moulton, K. S., Wu, H., Barnett, J., Parthasarathy, S., and Glass, C. K. (1992). Regulated expression of the human acetylated low density lipoprotein gene and isolation of promoter sequences. *Proc. Natl. Acad. Sci. USA* **89,** 8102–8106.

Moulton, K. S., Semple, K., Wu, H., and Glass, C. K. (1994). Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol. Cell. Biol.* **14,** 4408–4418.

Naito, M., Kodama, T., Matsumoto, A., Doi, T., and Takahashi, K. (1991). Tissue distribution, intracellular localization, and in vitro expression of bovine macrophage scavenger receptors. *Am. J. Pathol.* **139,** 1411–1423.

Naito, M., Suzuki, H., Mori, T., Matsumoto, A., Kodama, T., and Takahashi, K. (1992). Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. *Am. J. Pathol.* **141,** 591–599.

Nicoletti, A., Caligiuri, G., Tornberg, I., Kodama, T., Stemme, S., and Hansson, G. K. (1999). The macrophage scavenger receptor type A directs modified proteins to antigen presentation. *Eur. J. Immunol.* **29,** 512–521.

Palecanda, A., Paulauskis, J., Al Mutairi, E., Imrich, A., Qin, G., Suzuki, H., Kodama, T., Tryggvason, K., Koziel, H., and Kobzik, L. (1999). Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. *J. Exp. Med.* **189,** 1497–1506.

Paresce, D. M., Ghosh, R. N., and Maxfield, F. R. (1996). Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron* **17,** 553–565.

Pearson, A. M. (1996). Scavenger receptors in innate immunity. *Curr. Opin. Immunol.* **8,** 20–28.

Pearson, A. M., Rich, A., and Krieger, M. (1993). Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *J. Biol. Chem.* **268,** 3546–3554.

Peiser, L., Gough, P. J., Kodama, T., and Gordon, S. (2000). Macrophage class A scavenger receptor-mediated phagocytosis of *Escherichia coli:* Role of cell heterogeneity, microbial strain, and culture conditions in vitro. *Infect. Immun.* **68,** 1953–1963.

Platt, N., and Gordon, S. (1998). Scavenger receptors: Diverse activities and promiscuous binding of polyanionic ligands. *Chem. Biol.* **5,** R193–R203.

Platt, N., Suzuki, H., Kurihara, Y., Kodama, T., and Gordon, S. (1996). Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc. Natl. Acad. Sci. USA* **93,** 12456–12460.

Platt, N., da Silva, R. P., and Gordon, S. (1998). Recognizing death: The phagocytosis of apoptotic cells. *Trends Cell Biol.* **8,** 365–372.

Platt, N., Haworth, R., da Silva, R. P., and Gordon, S. (1999). Scavenger receptors and the phagocytosis of bacteria and apoptotic cells. *In* Phagocytosis: The Host pp. 71–85. Ed S. Gordon. Advances in Cell and Molecular Biology of Membranes and Organelles. Vol. 5. JAI, Stamford, CT.

Platt, N., Suzuki, H., Kodama, T., and Gordon, S. (2000). Apoptotic thymocyte clearance in scavenger receptor class A-deficient mice is apparently normal. *J. Immunol.* **164,** 4861–4667.

Porcelli, S. A., and Modlin, R. L. (1999). The CD1 system: Antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu. Rev. Immunol.* **17,** 297–351.

Redl, H., Bahrami, S., Schlag, G., and Traber, D. L. (1993). Clinical detection of LPS and animal models of endotoxaemia. *Immunobiology* **187,** 330–339.

Resnick, D., Pearson, A., and Krieger, M. (1994). The SRCR superfamily: A family reminiscent of the Ig superfamily. *Trends Biochem. Sci.* **19,** 5–8.

Resnick, D., Chatterton, J. E., Schwartz, K., Slayter, H., and Krieger, M. (1996). Structures of class A macrophage scavenger receptors. Electron microscopic study of flexible, multidomain, fibrous proteins and determination of the disulfide bond pattern of the scavenger receptor cysteine-rich domain. *J. Biol. Chem.* **271,** 26924–26930.

Robbins, A. K., and Horlick, R. A. (1998). Macrophage scavenger receptor confers an adherent phenotype to cells in culture. *Biotechniques* **25,** 240–244.

Rohrer, L., Freeman, M., Kodama, T., Penman, M., and Krieger, M. (1990). Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. *Nature* **343,** 570–572.

Ross, R. (1999). Atherosclerosis—An inflammatory disease. *N. Engl. J. Med.* **340,** 115–126.

Ruan, X. Z., Varghese, Z., Powis, S. H., and Moorhead, J. F. (1997). Human mesangial cells express inducible macrophage scavenger receptor: An Ap-1 and ets mediated response. *Kidney Int. Suppl.* **71,** S163–S166.

Sakaguchi, H., Takeya, M., Suzuki, H., Hakamata, H., Kodama, T., Horiuchi, S., Gordon, S., van der Laan, L. J., Kraal, G., Ishibashi, S., Kitamura, N., and Takahashi, K. (1998). Role of macrophage scavenger receptors in diet-induced atherosclerosis in mice. *Lab. Invest.* **78,** 423–434.

Sakai, M., Miyazaki, A., Hakamata, H., Sasaki, T., Yui, S., Yamazaki, M., Shichiri, M., and Horiuchi, S. (1994). Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidised low density lipoprotein on murine macrophages. *J. Biol. Chem.* **269,** 31430–31435.

Savill, J. (1997). Recognition and phagocytosis of cells undergoing apoptosis. *Br. Med. Bull.* **53,** 491–508.

Shackelford, R. E., Misra, U. K., Florine-Casteel, K., Thai, S.-F., Pizzo, S. V., and Adams, D. O. (1995). Oxidised low density lipoprotein suppresses activation of NKκB in macrophages via a pertussis toxin-sensitive signalling mechanism. *J. Biol. Chem.* **270,** 3475–3478.

Singh, N., Bhatia, S., Abraham, R., Basu, S. K., George, A., Bal, V., and Rath, S. (1998). Modulation of T cell cytokine profiles and peptide–MHC complex availability in vivo by delivery to scavenger receptors via antigen maleylation. *J. Immunol.* **160,** 4869–4880.

Stossel, T. P. (1999). The early history of phagocytosis. In Phagocytosis: The Host pp. 3–18. Ed. S. Gordon. Advances in Cell and Molecular Biology of Membranes and Organelles. Vol. 5. JAI, Stamford, CT.

Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., Kodama, T., *et al.,* (1997). A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* **386,** 292–296.

Suzuki, K., Doi, T., Imanishi, T., Kodama, T., and Tanaka, T. (1999). Oligonucleotide aggregates bind to the macrophage scavenger receptor. *Eur. J. Biochem.* **260,** 855–860.

Terpstra, V., Kondratenko, N., and Steinberg, D. (1997). Macrophages lacking scavenger receptor A show a decrease in binding and uptake of acetylated low-density lipoprotein and of apoptotic thymocytes, but not of oxidatively damaged red blood cells. *Proc. Natl. Acad. Sci. USA* **94,** 8127–8131.

Thomas, C. A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S. C., and El Khoury, J. (2000). Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. *J. Exp. Med.* **191,** 147–156.

Ulevitch, R. J., and Tobias, P. S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* **13,** 437–457.

Ulevitch, R. J., and Tobias, P. S. (1999). Recognition of gram-negative bacteria and endotoxin by the innate immune system. *Curr. Opin. Immunol.* **11,** 19–22.

Van Berkel, T. J., Van Velzen, A., Kruijt, J. K., Suzuki, H., and Kodama, T. (1998). Uptake and catabolism of modified LDL in scavenger-receptor class A type I/II knock-out mice. *Biochem. J.* **331,** 29–35.

Van Lenten, B. J., and Fogelman, A. M. (1992). Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor-alpha. *J. Immunol.* **148,** 112–126.

Van Lenten, B. J., Fogelman, A. M., Seager, J., Ribi, E., Haberland, M. E., and Edwards, P. A. (1985). Bacterial endotoxin selectively prevents the expression of scavenger-receptor activity on human monocyte-macrophages. *J. Immunol.* **134,** 3718–3721.

Van Velzen, A. G., Suzuki, H., Kodama, T., and Van Berkel, T. J. (1999). The role of scavenger receptor class A in the adhesion of cells is dependent on cell type and cellular activation state. *Exp. Cell Res.* **250,** 264–271.

Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W., and Stanley, E. R. (1990). Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc. Natl. Acad. Sci. USA* **86,** 2839–2843.

Witte, O. N. (1990). Steel locus defines new multipotent growth factor. *Cell* **63,** 5–6.

Wright, S. D. (1995). CD14 and innate recognition of bacteria. *J. Immunol.* **155,** 6–8.

Wu, H., Moulton, K., Horvai, A., Parik, S., and Glass, C. K. (1994). Combinatorial interactions between AP-1 and ets domain proteins contribute to the developmental regulation of the macrophage scavenger receptor gene. *Mol. Cell. Biol.* **14,** 2129–2139.

Yokota, T., Ehlin-Henriksson, B., and Hansson, G. (1998). Scavenger receptors mediate adhesion of activated B lymphocytes. *Exp. Cell Res.* **239,** 16–22.

Zhang, H., Yang, Y., and Steinbrecher, U. P. (1993). Structural requirements for the binding of modified proteins to the scavenger receptor of macrophages. *J. Biol. Chem.* **268,** 5535–5542.

Zimmerli, S., Edwards, S., and Ernst, J. D. (1996). Selective receptor blockade during phagocytosis does not alter the survival and growth of *Mycobacterium* tuberculosis in human macrophages. *Am. J. Respir. Cell. Mol. Biol.* **15,** 760–770.

Microtubule Transport in the Axon

Peter W. Baas
Department of Neurobiology and Anatomy, MCP Hahnemann University, Philadelphia, Pennsylvania 19129

There has been a great deal of interest in how the microtubule array of the axon is established and maintained. In an early model, it was proposed that microtubules are actively transported from the cell body of the neuron down the length of the axon. This model has been contested over the years in favor of very different models based on stationary microtubules. It appears that a corner has finally been turned in this long-standing controversy. It is now clear that cells contain molecular motor proteins capable of transporting microtubules and that microtubule transport is an essential component in the formation of microtubule arrays across many cells types. A wide variety of cell biological approaches have provided strong indirect evidence that microtubules are indeed transported within axons, and new live-cell imaging approaches are beginning to permit the direct visualization of this transport. The molecules and mechanisms that transport microtubules within axons are also under intense study.

KEY WORDS: Microtubule, Axon, Neuron, Axonal transport, Motor protein, Cytoplasmic dynein, Kinesin.

I. Introduction

Microtubules (MTs) form the infrastructure of eukaryotic cells, acting as both architectural elements and railways for the transport of cytoplasmic constituents. To serve these functions, MTs must be organized into a wide variety of configurations, ranging from the bipolar conformation of the mitotic spindle to the dense paraxial arrays that occupy elongated cellular processes. Thus, an important question in cell biology is how different cell types organize their MTs into these various configurations. Traditionally, cell biologists have focused on attachment to structures such as the centrosome as the means by which MTs are organized within the

cytoplasm (Brinkley, 1985). The centrosome nucleates a radial array of MTs with their plus ends outward and their minus ends inward. This mechanism can explain how MTs are organized in many simple interphase cell types. However, it cannot explain how MTs are organized into more sophisticated patterns such as those of the mitotic spindle or the elongated processes extended by cells such as neurons and glia. It appears that a variety of different mechanisms contribute to the elaboration of cellular MT arrays. Specific proteins have been identified which regulate the length, distribution, dynamic properties, and polarity orientation of the MTs as well as the manner by which they associate with one another and other structures in the cytoplasm (McNally, 2000; Heald, 2000; Andersen, 2000; Walczak, 2000; Sharp *et al.,* 2000a).

One of the most compelling ideas of how cells organize their MTs was proposed almost three decades ago on the basis of studies on axonal transport in neurons. These studies showed that proteins are actively transported down axons in discrete rate components, thus suggesting that they are conveyed as macromolecular complexes as opposed to individual protein subunits (Lasek, 1988; Baas and Brown, 1997; Brown, 2000). On this basis, it was suggested that tubulin is actively transported down the axon in the form of MT polymers (rather than as free tubulin), and hence that axons must contain "molecular machinery" capable of accomplishing this. It is not difficult to imagine how such machinery might be specialized to transport a MT specifically with either its plus end or its minus end leading and thereby establish distinct patterns of MT polarity orientation (Baas and Ahmad, 1993). There was little discussion in the early work as to whether MT transport is a neuron-specific mechanism or whether it might function across cell types, but during the past decade there has been an explosion of new information relevant to this issue. It is now clear that cells contain enzymes called molecular motor proteins which generate forces that can transport and configure MTs (Vale, 1999). The best studied example of this is the mitotic spindle, which has been shown to require motor-driven transport of MTs for its formation and its transitions from one stage to another (Sharp *et al.,* 1999, 2000b). The transport of MTs has been directly visualized during mitosis (Sharp *et al.,* 2000c) and also within relatively simple interphase cells (Keating *et al.,* 1997; Yvon and Wadsworth, 2000). Today, it is difficult to imagine MT research without live-cell images of MTs moving across video monitors.

The great irony is that although MT transport was first appreciated in axons and is arguably most important in axons given their great length, the issue of whether MTs move has been most controversial in axons. For reasons that are mostly either historical or technical, the view that MTs move down the axon has been repeatedly challenged throughout the years, with strong voices arguing that axonal MTs do not move. During the 1990s a flurry of papers appeared from opposing camps, some of which trumpeted that axonal MTs definitely do not move (Hirokawa *et al.,* 1997) and others of which trumpeted that they definitely do move (Baas and Brown, 1997). Today, it appears that a corner has been turned, and there are only a few

voices still arguing that axonal MTs are stationary. Although the end of any good controversy is a sad moment for its participants, this controversy leaves in its wake an exciting future of observational and mechanistic studies on MT transport in the axon. The goals of this article are to discuss the history of the MT transport controversy, to present the strong evidence that MTs are indeed transported down axons, and to explore new frontiers for understanding the mechanisms that orchestrate and regulate MT transport in the axon.

II. General Background

A. Early History of the Microtubule Transport Controversy

The theory of MT transport in axons was first proposed on the basis of classic axonal transport studies conducted over two decades ago. In these studies, radiolabeled amino acids were introduced into a cluster of cell bodies in which they were rapidly taken up and utilized for protein synthesis (Lasek, 1988; Baas and Brown, 1997; Brown, 2000). The labeled amino acids were presented for a brief period of time, thus creating a pulse of labeled proteins that could then move down the axon with a leading edge and a trailing edge (Fig. 1). After some time, the axon was cut into pieces that could be analyzed for radiolabeled protein content. Using this technique, it was determined that tubulin, the protein that constitutes MTs, is actively transported down the axon in the phase of axonal transport known as the "slow component" at an average rate of about 1 mm per day. The slow component could be further resolved into two subcomponents known as "slow component a" and "slow component b." Most of the tubulin was found in a, which is the slower moving of the two. The "pulse" of labeled tubulin moved down the axon relatively coherently at first but clearly spread as it moved, indicating a divergence in the rates of movement within the population. Initial considerations focused on the coherence and proposed that tubulin is transported in the form of a highly cross-linked network of MTs. Subsequent considerations took into account the spread and proposed that tubulin is more likely transported in the form of individual MTs that move at a variety of rates. In this modified view the rate of tubulin movement in the slow component represents an average rate of the movement of large numbers of individual MT polymers. In this view, the fastest moving MTs (those at the leading edge) move many times the average rate, at speeds comparable to the "fast transport" of membranous vesicles along MTs. These earlier studies largely predated the so-called "motor revolution"; hence, the movement of MTs was usually discussed in terms of hypothetical "transport machinery" rather than molecular motor proteins.

The first significant challenge to this polymer-transport model came from studies showing that MT poisons stopped axonal growth in culture when applied to the

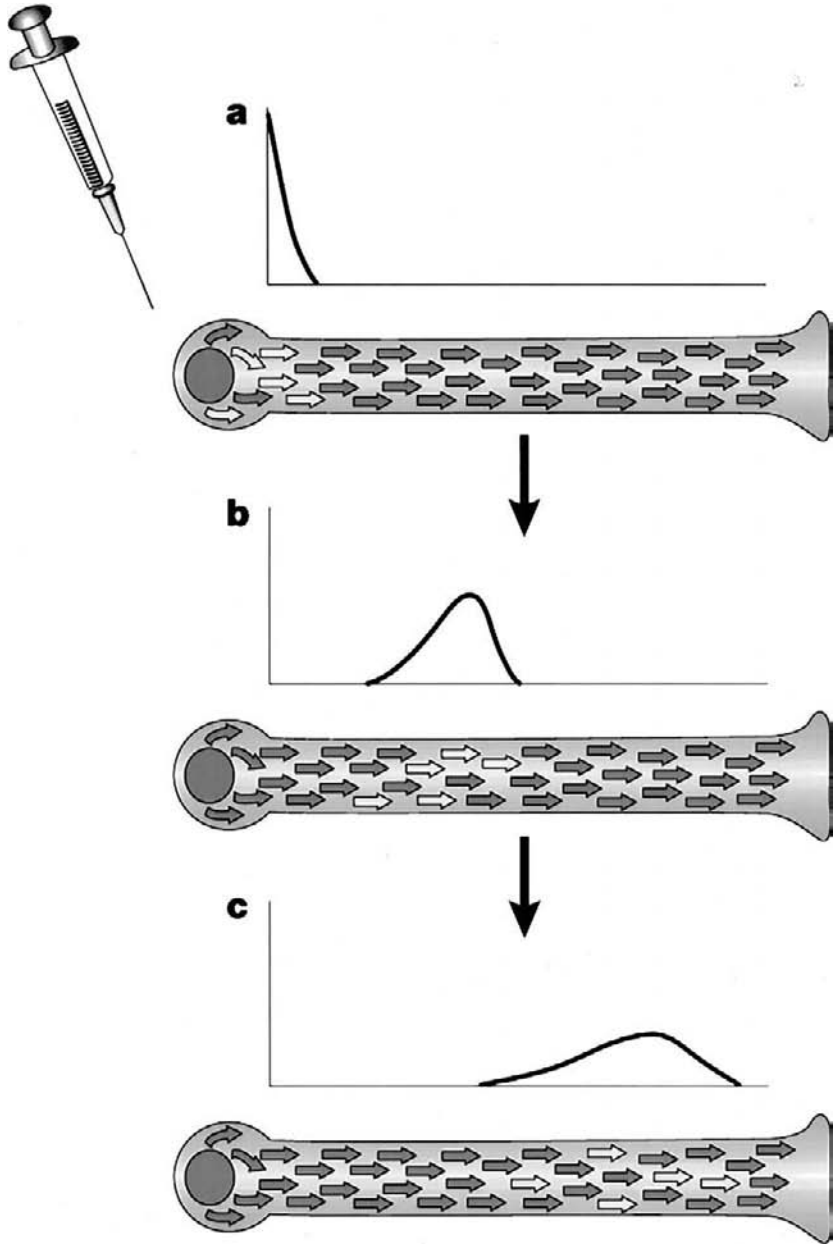


FIG. 1 Schematic illustration of the classic experimental paradigm for studying axonal transport of proteins. Radiolabeled amino acids are introduced near clusters of cell bodies, where they are taken up, incorporated into proteins, and then actively transported down the axon. A moving wave of labeled proteins can be detected. With time, the wave becomes broader [reprinted by permission from *Nature Reviews Molecular Cell Biology* (Brown, 2000) copyright 2000 Macmillan Magazines Ltd.].

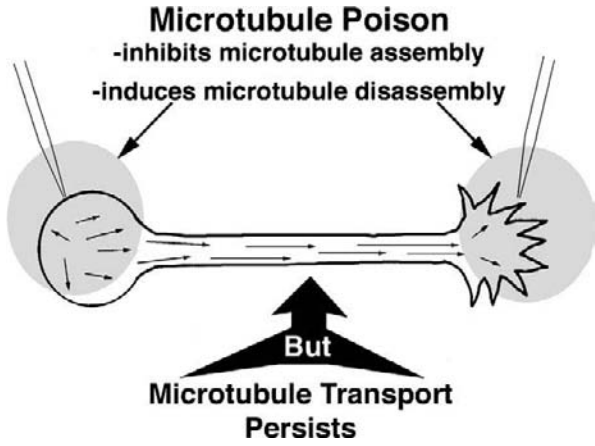


FIG. 2 Schematic illustration showing that application of microtubule poisons locally at the cell body of the neuron or the axon tip would produce local microtubule disassembly and curtail new microtubule assembly but would not inhibit the movement of microtubules down the axon.

distal tip of the axon but not to the cell body (Bamburg *et al.,* 1986). These studies were enormously influential, but in fact were based on a misconception of MT transport. The axon is full of MTs that are shorter than the length of the axon; being dynamic structures, these MTs can elongate and shorten. The transport of a MT is the actual movement of the polymer through the cytoplasm and is not contingent on the assembly of new polymer. Therefore, the relative sensitivity of axonal growth to MT poisons applied at the cell body or the axon tip is not directly relevant to whether MTs move down the axon (Fig. 2). Other studies have also confused these issues and have mistakenly taken evidence for local MT assembly in axons as evidence that MTs do not move. Subsequent studies sought to clear up this confusion and demonstrated that axons grow and MTs appear in new regions of axonal growth under conditions in which MT assembly is inhibited and even under conditions in which there is a net disassembly of MTs (Baas and Ahmad, 1993; Yu and Baas, 1995). A simple analogy is a marching army that can certainly continue to move regardless of whether or not new soldiers are added and even when soldiers are lost.

The idea that MTs do not have to move because they can assemble was problematic from the start. A fundamental challenge for axons is that they are incapable of protein synthesis and yet grow to great lengths that cannot be supplied simply by diffusion of proteins manufactured in the cell body. Therefore, energy-dependent mechanisms must exist to actively transport molecules down the axon. Tubulin subunits can certainly diffuse as well as any soluble molecule their size might be expected to diffuse, but this would not be sufficient to deliver enough tubulin fast enough to support axonal growth or to maintain the axon after it has stopped

growing (Sabry *et al.,* 1995). In the late 1980s and through the 1990s, the question became more tightly focused: We know that tubulin must somehow be actively transported down the axon, but what is the form in which it is actively transported? Microtubules were one clear option, but the view that MTs move down axons still seemed counterintuitive to many people who preferred the idea of moving subunits and stationary polymers.

B. The First Round of Live-Cell Analyses for Microtubule Transport

In the late 1980s, a microscopic strategy was developed to directly visualize whether or not MT polymers are transported down the axon. In this approach, fluorescent or caged-fluorescent tubulin subunits are introduced into cultured neurons and then permitted to incorporate into MTs throughout the neuron. A short segment of the axon is then marked by photobleaching or photoactivating the tagged tubulin. In the photobleach approach, the MTs within the neuron are fluorescent, but the regions of MTs in the marked zone are bleached and hence lose their fluorescence. In the photoactivation approach, the tagged tubulin does not fluoresce until it is activated; hence, only the regions of MTs in the marked zone are fluorescent. In either case, the next step is to capture images of the marked segment every few minutes and search for movement of the marked zone down the axon. Notably, almost all these studies failed to reveal any movement of the marked MT regions (Hirokawa *et al.,* 1997). The major exception was a study performed on the axons of frog motor axons, in which coherent movement of the marked zone was consistently observed (Reinsch *et al.,* 1991). However, a subsequent study suggested that this movement might somehow reflect passive dragging of the MTs related to the pulling of the growth cone rather than bona fide motor-driven MT transport (Chang *et al.,* 1998).

Assuming that the photomark technique does not artifactually immobilize MTs that are normally in motion, what can be concluded from the results of these studies? The clearest point made by these studies is that MTs within the axon (at least most axons) do not move synchronously. In order for the photomark method to reveal transport, the MTs in the marked zone would have to move essentially in unison down the axon (which was indeed the original way in which MT transport was conceived). Each MT would have to move at approximately the same rate and in the same anterograde direction if the photomarked region were to march coherently down the axon. The results of the photomark studies are important for dispelling this idea (although, as noted previously, careful consideration of the data from the earlier radiolabel work suggested a broad range of transport rates for MTs). In addition, the fact that the photomarked region remains stationary for lengthy periods of time suggests that many or even most of the MTs in the axon are not in motion at any given time. However, the photomark results never

disproved that MTs move down the axon. Other authors consistently pointed out that if MTs move at different rates, stop and start, pause, and even potentially move in the retrograde direction at times, then it would be unlikely that the photomark approach (as used in these studies) would reveal such movements (Baas, 2000; Brown, 2000; Fig. 3).

C. Subunit Transport Models

The lack of movement observed in the photomark studies buoyed opponents of MT transport models and encouraged them to pursue models based on actively transported tubulin subunits. In these models, tubulin is transported either as individual dimeric subunits or as hypothetical clusters of subunits (sometimes referred to as oligomers). The idea that motor proteins would transport individual soluble tubulin molecules or even small clusters is immediately problematic because it would call for an incredibly huge number of motor molecules. Nevertheless, the fact that tubulin simply must be actively transported in some form prompted the advent of experimental approaches that might reveal the active transport of tubulin dimers or oligomers.

Two very different experimental approaches were claimed to produce data supporting subunit transport models. In one of them, fluorescent tubulin was microinjected into cultured neurons in the presence of very low levels of a drug called vinblastine, which slows dynamic exchange of tubulin subunits (Miller and Joshi, 1996). Under these conditions, Miller and Joshi claimed to have documented a wave of incorporation of labeled tubulin into MTs which moves down a short axon of a cultured neuron with kinetics similar to the wave of moving radiolabeled tubulin in the classic axonal transport studies. However, it is not apparent why treatment with low levels of this drug should reveal such a phenomenon or how such a wave could be produced with a "pulse" but no "chase" in the experimental regime. No movement of anything was actually observed—only the incorporation of fluorescent subunits into MTs in different regions of the axon. Thus, these results are not directly relevant to the issue of MT transport (for further discussion, see Slaughter *et al.,* 1997). In the other approach, fluorescent tubulin was introduced into the squid giant axon and was noticed to diffuse both anterogradely and retrogradely (Terada *et al.,* 2000; Galbraith *et al.,* 1999). There was a slight bias of movement in the anterograde direction, which was said to correspond to the active transport of tubulin, superimposed on the bidirectional diffusive transport. This bias was obliterated by anti-MT drug treatments, which prompted the authors to conclude that free tubulin subunits or oligomers are actively transported along MTs. However, the authors neglected to take into account the fact that such a drug treatment would only partially depolymerize MTs, leaving MT "tracks" along which the hypothetical transport could occur. A more reasonable explanation for their finding is that the drug prevented the free tubulin from incorporating into MTs, which is the necessary

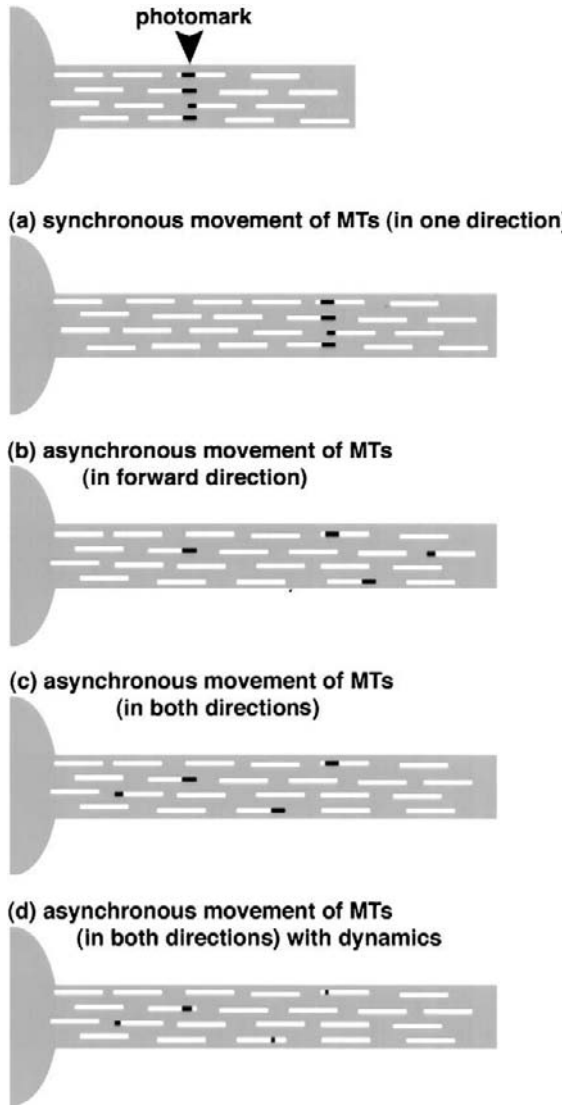


FIG. 3 Schematic illustration showing that the photomark approach (as utilized in the studies of the 1980s and 1990s) would likely detect microtubule transport if the microtubules move slowly and synchronously down the axon but probably would not have the sensitivity to detect microtubule transport if the microtubules move asynchronously and bidirectionally and undergo dynamics as they move (modified with permission from Baas, 2000).

form for it to be actively transported. In any event, the results of both of these approaches are inconclusive, and it is not at all obvious why they should be viewed as support for a subunit transport model as opposed to a polymer transport model.

III. Microtubule Transport as Revealed by Indirect Approaches

At approximately the same time as the previously mentioned studies were reported, other laboratories presented indirect data that favored the view that MTs are indeed transported down the axon. One of these approaches involved a more rigorous use of vinblastine as a tool to suppress MT dynamics. It was reasoned that at concentrations of the drug that more thoroughly suppress MT assembly and disassembly events, any redistribution of MTs that occurs over time could be attributed to the movement of MT polymers that had already been assembled prior to the introduction of the drug. In an early paper using this method, rat sympathetic neurons were cultured in the presence of various concentrations of the drug (Baas and Ahmad, 1993). Quantitative electron microscopy was used to ascertain a concentration of the drug that prohibited the total MT mass from increasing as well as individual MTs from elongating. For additional confidence, experiments were performed using concentrations of the drugs that were actually much higher—high enough to cause substantial MT disassembly. Even under these conditions, the existing MTs showed dramatic and consistent patterns of redistribution over time. The neurons still extended axons (although they were shorter and more branched) and there was a progressive loss of MTs from the cell body with a concomitant accumulation of MTs within the axons. These results suggest that MTs in the cell body are transported into developing axons. In addition, the MTs accumulated in the axons with the appropriate polarity orientation, providing the first evidence that it is the transport of the MTs (rather than how or where they assemble) that accounts for their uniformly plus end distal polarity pattern.

These studies demonstrated that axonal growth relies on an outward progression of MTs. In a subsequent paper, the vinblastine approach was used to document the outward progression of MTs from the centrosome (their site of nucleation) to the periphery of the neuronal cell body and into newly forming axons (Ahmad and Baas, 1995). In this study, freshly plated sympathetic neurons were treated with a high dose of another anti-MT drug called nocodazole in order to depolymerize most of the MT polymer. The drug was then rinsed from the culture for a few minutes to permit a burst of MT assembly from the centrosome, after which the vinblastine was added to suppress further MT assembly from occurring. Within 30 min, virtually all the MTs had left the centrosome and had accumulated at the periphery of the cell body, suggesting their active and directional transport outward. A very similar approach was taken by another laboratory to show that the

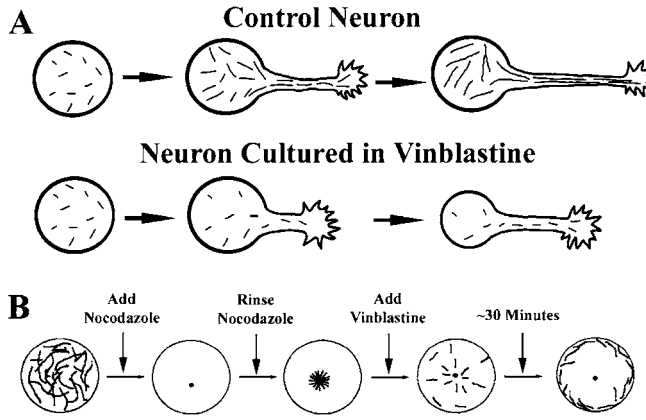


FIG. 4 Schematic illustrations of pharmacological studies which indirectly reveal microtubule transport in the neuron. When cells are cultured in the presence of low levels of vinblastine, microtubule assembly is suppressed, but axons grow nevertheless, and microtubules are redistributed from the cell body (A). A more complex pharmacologic regime demonstrates that microtubules nucleated at the centrosome are released and transported outward (B) (b is modified with permission from Ahmad *et al.,* 1998).

invasion of MTs into newly forming axonal branches is due to their transport from the parent axon (Gallo and Letourneau, 1999). Of course, the natural criticism against this approach is that drugs have complex modes of action, and one cannot be entirely certain that the vinblastine is having the desired effects. As a direct test of this (using the regime involving redistribution of MT within the cell body), fluorescent tubulin was introduced into the neurons at the time of vinblastine treatment (Ahmad *et al.,* 1998). If the MTs had assembled at the periphery of the cell body after the addition of the drug, they would have incorporated the fluorescent tubulin. However, there was no detectable incorporation of fluorescent tubulin during the redistribution of the polymer, indicating that the MTs must have been transported outward from the centrosome. Figure 4 summarizes the results of these pharmacological studies.

A major dilemma in visualizing MT transport is that MTs are dynamic structures that undergo dynamic assembly and disassembly events. At the same time that a MT may be moving, it might also be changing its length, and this leads to confusion in determining whether it is in fact moving. The vinblastine approach addresses this dilemma by suppressing MT dynamics so that changes in MT distribution can be attributed to their movement. Nevertheless, even with the studies indicating the reliability of the approach, it is important to develop other techniques that reveal MT transport without the use of pharmacologic agents. This was accomplished by using a labeling approach similar to the one described previously for proving that the vinblastine treatment did indeed reveal MT transport within the neuronal cell body.

Specifically, information was sought on whether MTs appeared in new regions of the axon prior to undergoing a bout of assembly or subunit turnover. If a MT appeared in a new region of the axon prior to such a bout, then it must have been transported into that region. This strategy was originally used to determine whether short MTs are transported from the parent axon into newly forming collateral branches (Yu *et al.,* 1994). In these analyses, the tyrosination state of the MTs was used as a

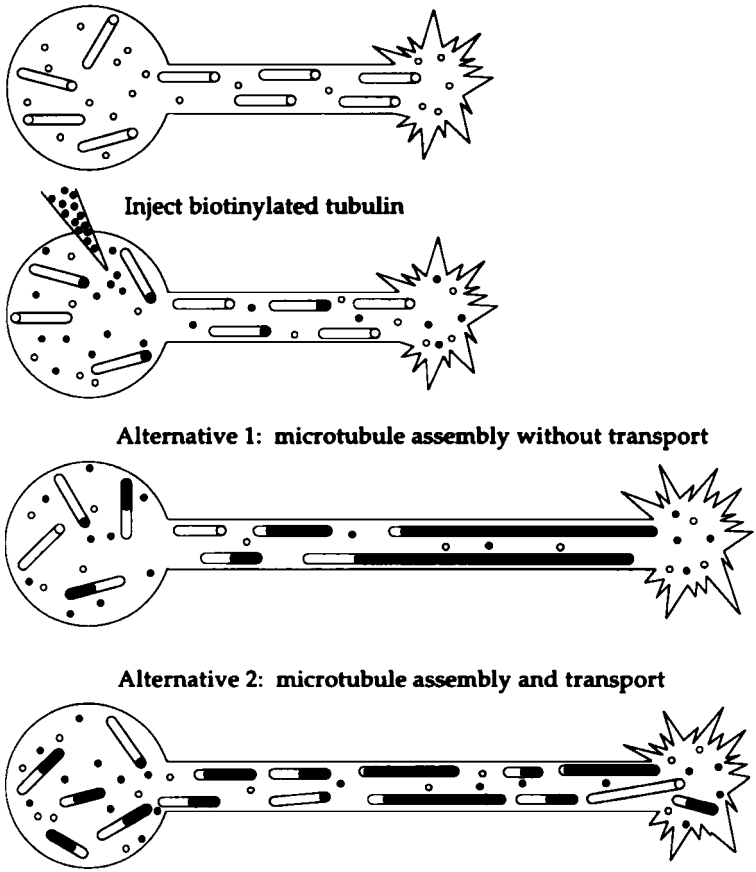


FIG. 5 Schematic illustration of a strategy for revealing microtubule transport. Neurons are injected with labeled tubulin subunits, and then the axon is permitted to grow longer. If microtubules assemble but do not move, all the microtubule polymer in the region of new growth should be labeled. If the microtubules undergo transport as well as assembly, then a portion of the microtubule polymer in the region of new growth should be unlabeled. Experimental studies reveal that the latter is the case during axonal growth (reproduced from *The Journal of Cell Biology*, 1996, Vol. 133, pp. 151–157, by copyright permission of The Rochefeller University Press).

marker for when they were assembled. The α-tubulin subunits within the MT are gradually detyrosinated after the polymer is assembled. Older MTs contain more detyrosinated α-tubulin, whereas younger MTs contain more tyrosinated α-tubulin. The MTs in newly forming collateral branches were similar in tyrosination state to those in the parent axon, indicating that these MTs were assembled in the parent axon and then transported into the new branch.

A more rigorous variation of this strategy was subsequently taken by two different laboratories (Yu *et al.,* 1996; Slaughter *et al.,* 1997). In these studies, biotinylated tubulin was microinjected into neurons either shortly after the outgrowth of axons or just prior to axonal outgrowth. The biotinylated tubulin was permitted to diffuse throughout the neuron, and the axons were permitted to grow. After a period of axonal growth, the distribution of biotinylated tubulin that had become incorporated into MTs was analyzed. The presence of labeled tubulin in MTs indicated assembly of new polymer or subunit exchange since the injection, so any unlabeled polymer that appeared within the newly grown region of the axon must have been assembled prior to the injection and then moved into this region (Fig. 5). This approach was expected to underestimate the amount of transported polymer because some of the transported MTs could have also elongated or exchanged subunits. Nevertheless, varying amounts of unlabeled polymer were found in the newly grown regions of the axon. The unlabeled MTs were intermingled with labeled MTs, confirming that the labeled tubulin had reached the most distal regions of the axon. These studies demonstrate that MTs are transported anterogradely within the axon (Yu *et al.,* 1996) and that MTs are transported from the cell body of the neuron into the axon during its genesis (Slaughter *et al.,* 1997).

IV. Live-Cell Imaging Breakthrough

By the late 1990s, it was clear that a MT transport model was more likely than a subunit transport model. The indirect analyses previously described provided strong evidence favoring this view. The logic of the classic axonal transport studies remained strong. The motor revolution had long since arrived and it was clear that cells had the necessary machinery to move cytoskeletal polymers. Studies that did not reveal MT movement, namely the photomark studies, failed to reveal tubulin movement in any form and hence were inconclusive. Studies that purported to reveal active transport of tubulin subunits were confusing and open to other interpretations. Nevertheless, the nagging question persisted that if MTs are actively transported down the axon, why does this movement continue to evade detection in direct live-cell studies? In 1999, a paper was published in which a hopeful new approach was taken (Chang *et al.,* 1999). Very low levels of fluorescent tubulin were introduced into cultured neurons so that MTs became labeled unevenly

with bright patches called "speckles" intermingled with nonglowing regions along the length of the polymer. The speckles provided fiduciary marks that could be observed for movement of individual polymers. This overcame the shortcoming of the photomark approach, which tested only for groups of polymers moving synchronously. Again, observations were made every few minutes, and again no movement was detected. The authors noted that MT transport would have made sense because cells do have the machinery to move MTs, but they concluded on the basis of their results that MTs do not move down axons (for additional discussion, see Hollenbeck and Bamburg, 1999).

At approximately the same time, another paper provided an initial insight into why this approach failed to detect movement (Dent *et al.,* 1999). Cultured neurons were injected with fluorescent tubulin and attention was given to the broader, flatter regions of the axon as opposed to the thinner axonal shaft. These regions, namely growth cones and branch points, were sufficiently flat that individual MTs could be visualized, and many of the individual MTs were sufficiently short that both ends could be visualized simultaneously. Unlike in the photomark studies, attention was given to individual MTs rather than marked zones of multiple MTs, and images were obtained every several seconds rather than every few minutes. Microtubules were directly observed to move in these broad, flat regions. Interestingly, the movement was bidirectional and occurred at rates that were many times faster than the average rate of slow axonal transport (Fig. 6). Also of note, the authors observed many MTs which failed to move during the time frame of observation. The rate of movement seemed to be inversely proportional to the length of the MTs. Although these studies documented MT transport in growth cones and at branch points, the resolution was not sufficient to determine whether MTs were moving down the main shaft of the axon. Notably, an earlier study on growth cones in which kinks and bends were observed for motion also concluded that

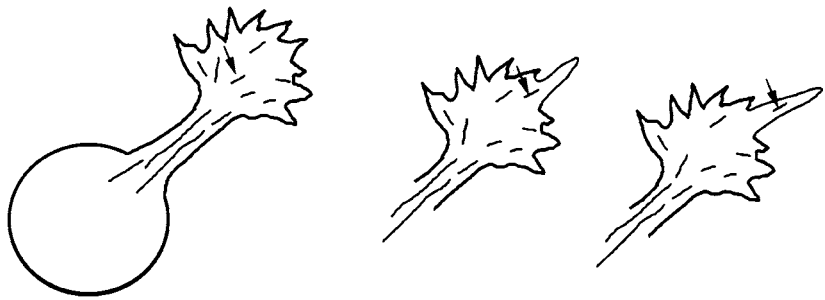


FIG. 6 Schematic illustration of live-cell imaging work (Dent *et al.,* 1999) of fluorescent microtubules moving within a flattened growth cone of a developing axon. Microtubules can be observed to move forward (arrows) as well as backward (note the microtubule to the left of the arrow in the first part of the illustration).

MTs move (Tanaka and Kirschner, 1991). Although it seems reasonable that the same machinery would be present to move MTs down the shaft, one could argue that growth cones and branch points are specialized in some way to accommodate MT transport.

Shortly after the Dent article was published, two critically important papers from two different laboratories were published (Wang *et al.,* 2000; Roy *et al.,* 2000). Notably, these papers were not on MTs, but their findings (together with the other available evidence) offer a clear and simple explanation for the conflicting results from the MT transport studies to date. In these papers, fluorescent neurofilament proteins were introduced or expressed in cultured sympathetic neurons. The axons of these neurons have a sparse array of neurofilaments, with natural transient "gaps" along the axon in which there are no neurofilaments. These gaps (which are similar in appearance to the photobleached regions in the earlier studies except that the gaps are much longer) were observed every several seconds rather than at the longer intervals used in every photomark study performed in the past. The authors observed no movement of the gap, but they observed clear movement of neurofilament polymers through the gap (Fig. 7). The movement was intermittent and very fast, similar to the rates of MT transport within growth cones and branch points. A small fraction of the movements occurred in the retrograde direction. These studies demonstrated that neurofilaments are actively transported as polymers and revealed clear and logical reasons why the original parameters of the photomark approach would never have detected the movement. The photomark studies tested for slow and coherent movement and not for movement that is fast, asynchronous, intermittent, and bidirectional. Notably, most of the neurofilaments were actually not observed to move during the time frame of the experiment, suggesting that the polymers spend most of their time in a "pausing" mode. These results suggest that the average rate of transport reflects a combination of fast movements and pauses and demonstrate that polymer transport in the axon is more like "stop and go traffic" than a "slow train" (Brown, 2000).

These experiments are easier to perform with neurofilaments than with MTs because the axons of cultured sympathetic neurons have natural gaps in the neurofilament array, because the neurofilaments are less dynamic than MTs, and because there is far less unassembled neurofilament protein than free tubulin in the axon. Nevertheless, new studies on MTs, taking into account the parameters used in the neurofilament work, will very likely reveal movement. Either the speckle or the photomark approach is probably necessary in light of the fact that there are no natural gaps in the MT array.

The beauty of the recent neurofilament studies is not only that they unequivocally demonstrate that polymers move within the axon but also that they provide a clear and satisfactory explanation for why the previous live-cell studies failed to reveal that movement. Had earlier studies using either the photomark or the speckle approach acquired images at shorter intervals, it seems almost certain that they would have documented polymers moving rapidly. Interestingly, in some of the

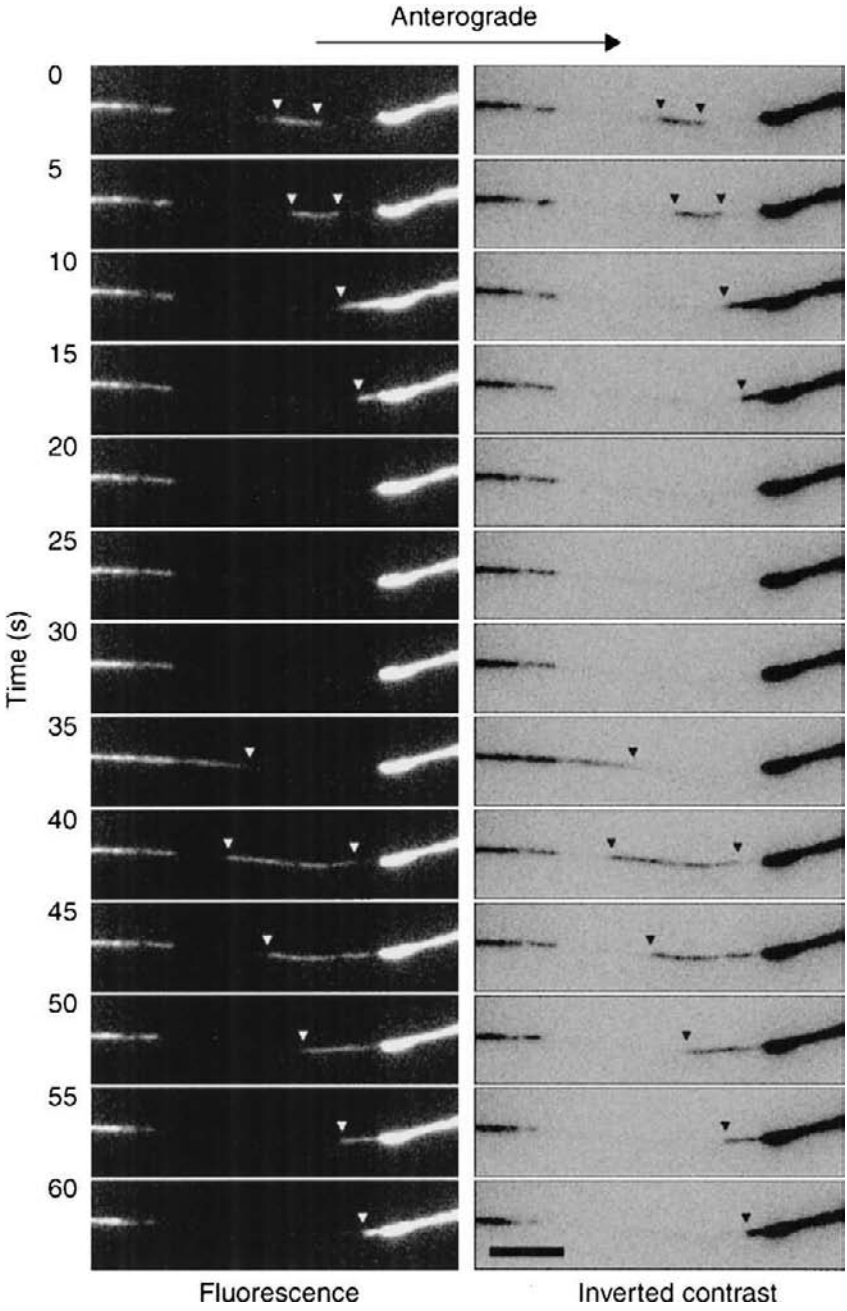


FIG. 7 Images of moving neurofilaments obtained by observing every few seconds a gap in the neurofilament array of axons from sympathetic neurons induced to express a fluorescent neurofilament protein. Scale bar $= 5\ \mu$m [reprinted by permission from *Nature Cell Biology* (Wang *et al.,* 2000) copyright 2000 Macmillan Magazines Ltd.].

earlier studies, the authors confessed that they would not have been able to detect movement if only 10–20% of the polymers were in motion at any given time (Lim *et al.,* 1990; Sabry *et al.,* 1995). However, they concluded that this amount of movement would not be sufficient to sustain growth of the axon. The oversight in this reasoning is that the rate of movement (when the polymers actually do move) was considered to be the average rate of slow transport rather than the significantly faster rates at which the polymers actually move (see also Campenot *et al.,* 1996). At these faster rates, a low frequency of MT movement would be perfectly sufficient. It is also worth noting that the idea of intermittent fast movements is entirely consistent with the more contemporary interpretation of the classic axonal transport work and fits well with the results of the various indirect analyses that strongly indicate that MTs must move.

V. The Machinery of Microtubule Transport in the Axon

Perhaps the more interesting question is not whether MTs move but how they move. What is the nature of the mysterious "transport machinery" that was proposed decades ago? The original conception, at least according to some workers, was that neurons contain a highly specialized set of tools for transporting MTs from the cell body down the length of the axon. Today, we know that cytoplasm is rich in a class of enzymes known as molecular motor proteins that hydrolyze ATP to move along the surface of the MT (Vale, 1999). Motor proteins consist of a motor domain that moves along the MT and a cargo domain that can interact with other cytoplasmic structures. If the cargo domain interacts with an organelle with less resistance to movement than the MT, this organelle will move along the surface of the MT. However, if the cargo domain interacts with a structure with more resistance to movement than the MT, then (all motion being relative) the MT will move. This has been demonstrated repeatedly by *in vitro* experiments in which motor proteins are immobilized on glass. During the past several years, enormous progress has been made in elucidating the role of force generation by motor proteins in the formation and functioning of the mitotic spindle. This has led to a fascinating view of how MTs are organized and distributed within the cytoplasm of cells; specifically, motor proteins push and pull MTs, integrate them relative to other structures in the cell, configure them, and orient them. All of this is accomplished by the capacity of the motor to move the MT. In light of all of this, a new perspective on axonal MTs has emerged. Perhaps the transport machinery that moves MTs down the axon is not so special but rather is a manifestation of the same kinds of fundamental mechanisms that organize and distribute MTs across cell types (Baas, 1999; Sharp *et al.,* 2000a).

In order to move MTs anterogradely down the axon, the relevant motor protein must be quite abundant along the length of the axon and must have the appropriate

properties to transport MTs with the plus end of the MT leading. Within the axon, all the MTs have their plus ends directed away from the cell body. Most of the kinesin superfamily members move toward the plus ends of MTs and hence would move MTs with their minus ends leading. On the other hand, cytoplasmic dynein moves toward minus ends of MTs, and hence would have the appropriate directionality to move MTs with their plus ends leading against a non-MT substrate. Initial support for the view that cytoplasmic dynein does in fact serve this function came from studies using a traditional axonal transport (radiolabel) approach. These studies showed that the vast majority of the cytoplasmic dynein that is anterogradely transported down the axon moves in the slow component of axonal transport (Dillman *et al.,* 1996). Interesting, cytoplasmic dynein moves in slow component b rather than slow component a, whereas MTs can be detected in a or b. The principal cytoskeletal elements in slow component b are microfilaments, which are composed of actin. On the basis of these observations, it was proposed that the cargo domain of cytoplasmic dynein interacts with the actin cytomatrix of the axon and moves down the axon by virtue of the fact that the actin cytomatrix is moving (presumably via myosin motors). The motor domain of the cytoplasmic dynein is available for intermittent interactions with MTs. When MTs engage the motor, they can be rapidly moved anterogradely by pushing against the actin cytomatrix. There is enormous resistance to retrograde movement of the actin cytomatrix (both because it is highly cross-linked and because of the myosin-forces moving it anterogradely) and hence the MT moves anterogradely. The intermittent nature of MT transport results from factors which regulate its frequency and the efficiency of its interaction with the motor. Experimental support for this model was provided by studies showing that disabling cytoplasmic dynein compromises axonal growth and curtails the movement of MTs from the centrosome into developing axons (Ahmad *et al.,* 1998).

This model is very satisfying because it takes advantage of machinery that cells had developed prior to the evolution of neurons. During mitosis, a portion of the MTs nucleated by the centrosome emanate toward the cell periphery and interact with the actin-rich cell cortex. These MTs are called astral MTs. Cytoplasmic dynein generates forces between the astral MTs and the cortex, and these forces are important for drawing apart the duplicated centrosomes during anaphase. A similar phenomenon occurs in motile interphase cells; some of the MTs emanating away from the centrosome interact with the cell cortex, where forces are generated that drag the centrosome to a new location between the nucleus and the leading edge of the moving cell. It has been proposed that during axogenesis the same machinery is invoked, except that the MTs lose their association with the centrosome and hence do not drag the centrosome toward the cell cortex. Instead, the "free" MTs move into a newly forming axon that elongates between the stationary cell body and the motile leading edge (Baas, 1996; Fig. 8). Presumably, in neurons additional factors enhance the efficiency of the dynein-based transport machinery to ensure a continuous supply of moving MTs from the centrosome down the length of

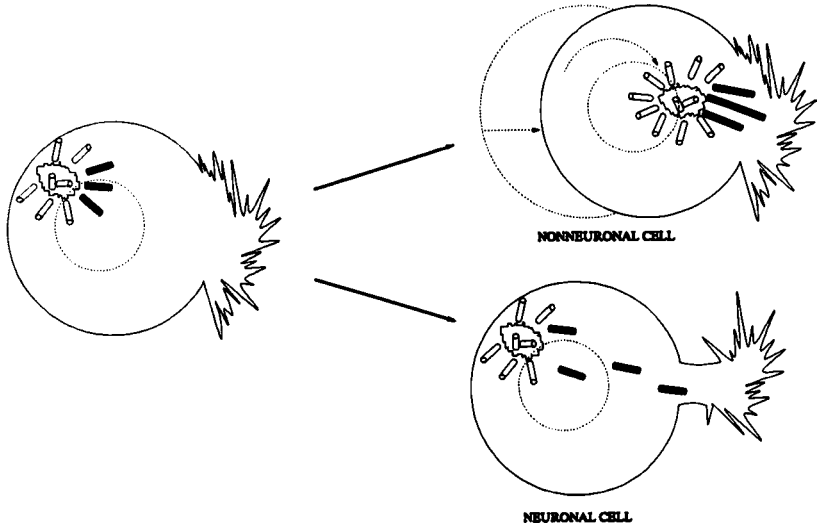


FIG. 8 Schematic illustration showing a key difference between a neuronal cell and a classic motile nonneuronal cell. In both cases, motor-driven forces draw microtubules toward the leading edge (by interacting with the cell cortex). In the nonneuronal cell, the microtubules remain associated with the centrosome, which is then relocated together with the microtubules toward the motile leading edge of the cell. In the neuron, the microtubules are completely detached from the centrosome and are conveyed independently of the centrosome toward the motile leading edge and continue moving down the developing axon as it forms between the cell body and the leading edge, which becomes the growth cone (reproduced with permission from Baas, 1996).

the axon. For example, the protein Lis1 is highly enriched in neurons compared to other cells, interacts with cytoplasmic dynein, and dramatically increases the efficiency of the outward progression of MTs (Smith *et al.,* 2000).

In theory, cytoplasmic dynein could be the only motor protein necessary to transport MTs down the axon. Cytoplasmic dynein could also move MTs retrogradely, given that the motor could move MTs against other MTs as well as the actin cytomatrix. However, it has been proposed that other motors might also contribute to the movement of MTs in the axon. The mitotic spindle utilizes a vast array of complementary and antagonistic motor-driven forces, and perhaps this is also true of the axonal cytoskeleton. Specific kinesin-related proteins have been identified in the mitotic spindle which are designed not for vesicle transport but specifically for force generation between MTs and other cytoskeletal elements. These kinesin-related proteins were initially thought to be mitosis specific because they appeared to be inactive during interphase. However, recent studies have revealed that these motors continue to be expressed in terminally postmitotic neurons, where they are prominent components of the MT arrays of axons or dendrites. The best studied example is CHO1/MKLP1, originally discovered in the midzonal region of the mitotic spindle in which MTs of opposite polarity orientation overlap. This motor

was found to be necessary for the transport of a population of MTs into dendrites oriented opposite to those found in the axon (Sharp *et al.,* 1997). There is no detectable CHO1/MKLP1 in the axon, but other recent studies have demonstrated that axons contain another mitotic motor protein called Eg5, which can also generate strong forces on MTs (Ferhat *et al.,* 1998). It is fascinating to consider that motors such as Eg5 (and other unexplored "mitotic kinesins") might generate forces on MTs in different regions of the axon. These forces could help drive MTs forward or backward, or they could potentially zipper together MT bundles or splay apart already formed bundles. These forces might also further integrate MTs with other cytoskeletal elements such as the microfilament system in different regions of the axon. Modulation of these forces could be an important factor in regulating the rate, frequency, and direction of MT transport.

Recent studies have led to the idea that MT transport is more than just the unidirectional movement of MTs from the cell body down the axon for the purpose of replenishing the MT supply. In this newer view, the machinery that transports MTs is more complex and flexible than that in previous models and can be regulated to play critical roles in plastic events, such as axonal retraction and branch formation. For example, the dynein-driven transport of MTs against the actin cytomatrix appears to attenuate the contractility of the actomyosin system that would otherwise tend to cause the axon to retract (Ahmad *et al.,* 2000). Increases or decreases in dynein or myosin-driven forces could thereby shift an elongating axon to a bout of retraction or a retracting axon back to elongating. Various other motors such as Eg5 might play a role in augmenting either the forward or the backward movement of MTs in particular regions of the axon. In this manner, alterations in motor-driven forces can be shifted to favor either axonal elongation or retraction, and such alterations might be tightly regulated by environmental factors that are key to the development of the nervous system (Baas and Ahmad, 2001; Fig. 9; see color insert). Interstitial branching appears to involve an increase in MT motile activity, which is linked to a fragmentation of MTs into shorter pieces (Yu *et al.,* 1994; Dent *et al.,* 1999). Perhaps activation of MT severing proteins such as katanin, known to be present within the axon (Ahmad *et al.,* 1999), could determine how readily the transport machinery can actually cause a MT to move. Traditional MT-associated proteins (MAPs), such as tau and MAP1b, might also assist in drawing together and aligning with the MTs other important elements of the "machine," such as the microfilament tracks that appear to be so critical for normal MT transport to occur.

VI. Concluding Remarks

Scholarly disagreement and debate over whether MT transport in the axon is real or mythical have occurred for decades. There now appears to be overwhelming evidence that MTs are in fact transported within the axon. New information about

the rates and frequencies of these movements and improved technologies are beginning to permit researchers to visualize these movements. In addition, it has become quite apparent that the machinery needed to transport MTs is present within neurons. Molecular motors and related molecules exist across cell types and are essential for transporting and configuring MTs in fundamental processes such as mitosis. Given all that is known today about motor-driven MT transport in other cell types, it would be a far stretch of the imagination to believe that neurons, arguably the cell type that needs most to transport its MTs, would simply turn off all the available machinery to do so. Even so, there are undoubtedly still many surprises for researchers in this field. Future efforts should prove exciting indeed, as researchers seek to visualize MT movements with greater clarity and to elucidate the array of molecules, signals, and mechanisms that regulate these movements.

Acknowledgments

Anthony Brown generously provided Figs. 1 and 7. Fridoon Ahmad prepared all the other figures. Anthony Brown, Mark Black, Dan Buster, and Yan He provided critical comments on the manuscript. The work in the author's laboratory is funded by two grants from the National Institutes of Health.

References

Ahmad, F. J., and Baas, P. W. (1995). Microtubules released from the neuronal centrosome are transported into the axon. *J. Cell Sci.* **108,** 2761–2769.

Ahmad, F. J., Echeverri, C. J., Vallee, R. B., and Baas, P. W. (1998). Cytoplasmic dynein and dynactin are required for microtubule transport into the axon. *J. Cell Biol.* **140,** 391–402.

Ahmad, F. J., Yu, W., McNally, F. J., and Baas, P. W. (1999). An essential role for katanin in severing microtubules in the neuron. *J. Cell Biol.* **145,** 305–315.

Ahmad, F. J., Hughey, J., Wittmann, T., Hyman, A., Greaser, M., and Baas, P. W. (2000). Motor proteins regulate force interactions between microtubules and microfilaments in the axon. *Nature Cell Biol.* **2,** 276–280.

Andersen, S. S. L. (2000). Spindle assembly and the art of regulating microtubule dynamics by MAPs and Stathmin/OP18. *Trends Cell Biol.* **10,** 261–267.

Baas, P. W. (1996). The neuronal centrosome as a generator of microtubules for the axon. *Curr. Topics Dev. Biol.* **33,** 281–298.

Baas, P. W. (1999). Microtubules and neuronal polarity: Lessons from mitosis. *Neuron* **22,** 23–31.

Baas, P. W. (2000). Strategies for studying microtubule transport in the neuron. *Microsc. Res. Techniques* **48,** 75–84.

Baas, P. W., and Ahmad, F. J. (1993). The transport properties of axonal microtubules establish their polarity orientation. *J. Cell Biol.* **120,** 1427–1437.

Baas, P. W., and Brown, A. (1997). Slow axonal transport: The polymer transport model. *Trends Cell Biol.* **7,** 380–384.

Baas, P. W., and Ahmad, F. J. (2001). Force generation by cytoskeletal motor proteins as a regulator of axonal elongation and retraction. *Trends Cell Biol.* **11,** 244–249.

Bamburg, J. R., Bray, D., and Chapman, K. (1986). Assembly of microtubules at the tip of growing axons. *Nature* **321,** 788–790.

Brinkley, B. R. (1985). Microtubule organizing centers. *Annu. Rev. Cell Biol.* **1,** 145–172.

Brown, A. (2000). Slow axonal transport: Stop and go traffic in the axon. *Nature Rev. Mol. Cell Biol.* **1,** 153–155.

Campenot, B., Lund, K., and Senger, D. L. (1996). Delivery of newly synthesized tubulin to rapidly growing distal axons of sympathetic neurons in compartmented cultures. *J. Cell Biol.* **135,** 701–709.

Chang, S., Rodionov, V. I., Borisy, G. G., and Popov, S. V. (1998). Transport and turnover of microtubules in frog neurons depend on the pattern of axonal growth. *J. Neurosci.* **18,** 821–829.

Chang, S., Svitkina, T. M., Borisy, G. G., and Popov, S. V. (1999). Speckle microscopic evaluation of microtubule transport in growing nerve processes. *Nature Cell Biol.* **1,** 399–403.

Dent, E. W., Szebenyi, G., Callaway, J. L., Baas, P. W., and Kalil, K. (1999). Reorganization and movement of microtubules in axonal growth cones and developing interstitial branches. *J. Neurosci.* **19,** 8894–8908.

Dillman, J. F., III, Dabney, L. P., and Pfister, K. K. (1996). Cytoplasmic dynein is associated with slow axonal transport. *Proc. Natl. Acad. Sci. USA* **93,** 141–144.

Ferhat, L., Cook, C., Chauviere, M., Harper, M., Kress, M., Lyons, G. E., and Baas, P. W. (1998). Expression of the mitotic motor protein Eg5 in postmitotic neurons: Implications for neuronal development. *J. Neurosci.* **18,** 7822–7835.

Galbraith, J. A., Reese, T. S., Shlief, M. L., and Gallant, P. E. (1999). Slow transport of unpolymerized tubulin and polymerized neurofilament in the squid giant axon. *Proc. Natl. Acad. Sci. USA* **96,** 11589–11594.

Gallo, G., and Letourneau, P. C. (1999). Different contributions of microtubule dynamics and transport to the growth of axons and collateral sprouts. *J. Neurosci.* **19,** 3860–3873.

Heald, R. (2000). A dynamic duo of microtubule modulators. *Nature Cell Biol.* **2,** E11–E12.

Hirokawa, N., Terada, S., Funakoshi, T., and Takeda, S. (1997). Slow axonal transport: The subunit transport model. *Trends Cell Biol.* **7,** 384–388.

Hollenbeck, P. J., and Bamburg, J. R. (1999). Axonal microtubules stay put. *Nature Cell Biol.* **7,** E171–E173.

Keating, T. J., Momcilovic, D., Rodionov, V. I., and Borisy, G. G. (1997). Microtubule release from the centrosome. *Proc. Natl. Acad. Sci. USA* **94,** 5078–5083.

Lasek, R. J. (1988). Studying the intrinsic determinants of neuronal form and function. In *Intrinsic Determinants of Neuronal Form and Function* (R. J. Lasek and M. M. Black, Eds.), pp. 1–58. A. R. Liss, New York.

Lim, S. S., Edson, K. J., Letourneau, P. C., and Borisy, G. G. (1990). A test of microtubule translocation during neurite elongation. *J. Cell Biol.* **111,** 123–130.

McNally, F. (2000). Capturing a ring of samurai. *Nature Cell Biol.* **2,** E4–E7.

Miller, K. E., and Joshi, H. C. (1996). Tubulin transport in neurons. *J. Cell Biol.* **133,** 1355–1368.

Reinsch, S. S., Mitchison, T. J., and Kirschner, M. W. (1991). Microtubule polymer assembly and transport during axonal elongation. *J. Cell Biol.* **115,** 365–380.

Roy, S., Coffee, P., Smith, G., Liem, R. K. H., Brady, S. T., and Black, M. M. (2000). Neurofilaments are transported rapidly but intermittently in axons: Implications for slow axonal transport. *J. Neurosci.* **20,** 6849–6861.

Sabry, J., O'Connor, T. P., and Kirschner, M. W. (1995). Axonal transport of tubulin in Ti1 pioneer neurons in situ. *Neuron* **14,** 1247–1256.

Sharp, D. J., Yu, W., Ferhat, L., Kuriyama, R., Rueger, D. C., and Baas, P. W. (1997). Identification of a motor protein essential for dendritic differentiation. *J. Cell Biol.* **138,** 833–843.

Sharp, D. J., Yu, K. R., Sisson, J. C., Sullivan, W., and Scholey, J. M. (1999). Antagonistic microtubule sliding motors position mitotic centrosomes in *Drosophila* early embryos. *Nature Cell Biol.* **1,** 51–54.

Sharp, D. J., Rogers, G. C., and Scholey, J. M. (2000a). Roles of motor proteins in building microtubule-based structures: A basic principle of cellular design. *Biochim. Biophys. Acta* **1496,** 128–141.

Sharp, D. J., Rogers, G. C., and Scholey, J. M. (2000b). Microtubule motors in mitosis. *Nature* **407,** 41–47.

Sharp, D. J., Brown, H. M., Kwon, M., Rogers, G. C., Holland, G., and Scholey, J. M. (2000c). Functional coordination of three mitotic motors in Drospholia embryos. *Mol. Biol. Cell* **11,** 241–253.

Slaughter, T. S., Wang, J., and Black, M. M. (1997). Transport of microtubules from the cell body into the axons of cultured neurons. *J. Neurosci.* **17,** 5807–5819.

Smith, D. S., Niethammer, M., Ayala, R., Zhou, Y., Gambello, M. J., Wynshaw-Boris, A., and Tsia, L.-H. (2000). Regulation of cytoplasmic dynein behavior and microtubule organization by mammalian Lis1. *Nature Cell Biol.* **2,** 767–775.

Tanaka, E. M., and Kirschner, M. W. (1991). Microtubule behavior in the growth cones of living neurons during axon elongation. *J. Cell Biol.* **115,** 345–363.

Terada, S., Kinjo, M., and Hirokawa, N. (2000). Oligomeric tubulin in large transporting complex is transported via kinesin in squid giant axons. *Cell* **103,** 141–155.

Vale, R. D. (1999). Millennial musings on molecular motors. *Trends Cell Biol.* **9,** M38–M42.

Walczak, C. E. (2000). Microtubule dynamics and tubulin interacting proteins. *Curr. Opin. Cell Biol.* **12,** 52–56.

Wang, L., Ho, C.-L., Sun, D., Liem, R. K. H., and Brown, A. (2000). Rapid movements of axonal neurofilaments interrupted by prolonged pauses. *Nature Cell Biol.* **2,** 137–141.

Yu, W., and Baas, P. W. (1995). The growth of the axon is not dependent upon net microtubule assembly at its distal tip. *J. Neurosci.* **15,** 6827–6833.

Yu, W., Ahmad, F. J., and Baas, P. W. (1994). Microtubule fragmentation and partitioning in the axon during collateral branch formation. *J. Neurosci.* **14,** 5872–5884.

Yu, W., Schwei, M. J., and Baas, P. W. (1996). Microtubule transport and assembly during axon growth. *J. Cell Biol.* **133,** 151–157.

Yvon, A. M., and Wadsworth, P. (2000). Region-specific microtubule transport in motile cells. *J. Cell Biol.* **151,** 1003–1012.

G Protein-Coupled Receptors: Dominant Players in Cell–Cell Communication

Joël Bockaert,* Sylvie Claeysen,* Carine Bécamel,* Sylvie Pinloche,† and Aline Dumuis*

*UPR CNRS 9023, 34094 Montpellier Cedex 5, France †LIRMM, 34392 Montpellier Cedex 5, France

The G protein-coupled receptors (GPCRs) are the most numerous and the most diverse type of receptors (1–5% of the complete invertebrate and vertebrate genomes). They transduce messages as different as odorants, nucleotides, nucleosides, peptides, lipids, and proteins. There are at least eight families of GPCRs that show no sequence similarities and that use different domains to bind ligands and activate a similar set of G proteins. Homo- and heterodimerization of GPCRs seem to be the rule, and in some cases an absolute requirement, for activation. There are about 100 orphan GPCRs in the human genome which will be used to find new message molecules. Mutations of GPCRs are responsible for a wide range of genetic diseases. The importance of GPCRs in physiological processes is illustrated by the fact that they are the target of the majority of therapeutical drugs and drugs of abuse.

KEY WORDS: G protein-coupled receptors, G proteins, Orphan receptors, Constitutive activation, Inverse agonism, Genetic diseases, Cellular signaling, Desensitization.

I. Introduction

In 1957, Sutherland and Rall were busy describing the basic properties of an enzyme, the adenylate cyclase—now called adenylyl cyclase (AC)—its activation by hormones such as epinephrine and glucagon, and its well-known product cAMP (Sutherland and Rall, 1958). Ten years later, AC was still believed to be a unique allosteric enzyme regulated by the binding of hormones on a regulatory site. In

the late 1960s, Rodbell and Birnbaumer demonstrated that fat cell AC is activated by multiple hormone receptors in a nonadditive manner, and they proposed that AC and hormonal receptors are distinct (Birnbaumer and Rodbell, 1969). This proposal was subsequently confirmed by Orly and Schramm (1976). Rodbell and Birnbaumer also demonstrated that receptors need a third partner to activate AC—the GTP-binding protein (G protein). G proteins play the go-between for receptors and AC (Rodbell *et al.,* 1971). In 1983, Gs (the G protein which activates the AC) was the first G protein to be purified by Gilman and coworkers (Northup *et al.,* 1983). Purification of rhodopsin, the G protein-coupled receptor (GPCR) activated by light, was achieved in 1967 (Shields *et al.,* 1967), whereas the first purification of a hormonal GPCR (the β-adrenergic receptor, β-AR) was carried out by Lefkowitz's team in 1981 (Shorr *et al.,* 1981).

Today, GPCRs constitute one of the largest known categories of proteins. Based on the fact that these receptors are all formed of seven-transmembrane domains (TM-I–TM-VII) and are believed to activate G proteins, GPCRs are generally assumed to form a single superfamily (Baldwin, 1993; Bockaert and Pin, 1998, 1999; Bourne, 1997b; Josefsson, 1999; Kolakowski, 1994; Wess, 1997). However, the more data we accumulate, the more complex the picture becomes, indeed, in this review, we demonstrate that (i) the comparison of primary amino acid sequences leads to the multiplication of GPCR families (up to eight families have been recognized to date) (Bockaert and Pin, 1999; Josefsson, 1999) and (ii) some GPCRs may signal by interacting with non-G proteins, with or without G protein signaling (Hall *et al.,* 1999). Calling some of them GPCRs may therefore be inaccurate. Many seven-transmembrane (TM) proteins, such as latrophilin (the receptor for the spider toxin α-latrotoxin), EMR1 (human cell surface glycoprotein F4/80), BAI-1 (brain-specific angiogenesis inhibitor), or Methuselah, classified as LNB-TM7 (seven TM domains containing a long N-terminal extracellular region), have been described (Stacey *et al.,* 2000). The long N-terminal domain of LNB-TM7 contains several repeats, such as EGF repeats, thrombospondin type 1 repeats, olfactomedin homologous regions, galactose-binding lectin homologous regions, and laminin AG-type repeats. If the coupling of LNB-TM7 proteins to G proteins occurs, it has only been demonstrated for latrophilin (Stacey *et al.,* 2000). Recently, another seven-transmembrane protein having 20–25% identity with the core domain of family 3 GPCRs, but devoid of the long and specific extracellular domain characteristic of this family, was cloned (Brauner-Osborne and Krogsgaard-Larsen, 2000). Whether or not it is a GPCR remains to be demonstrated. GPCRs clearly represent some of the oldest devices devoted to signal transduction, present in protozoa and the earliest diploblastic metazoa (New and Wong, 1998; Vernier *et al.,* 1995), slime mold (Devreotes, 1994), yeast (Dohlman *et al.,* 1991), plants (Josefsson and Rask, 1997; Plakidou-Dymock *et al.,* 1998), invertebrates (Bargmann, 1998; Rubin *et al.,* 2000), and vertebrates (Bockaert and Pin, 1999). GPCRs represent 5% *Caenorhabditis elegans* (1100 genes) (Bargmann, 1998), 1% *Drosophila* (160 genes) (Rubin *et al.,* 2000), and 1–3% of vertebrate genomes (more than 1000 genes) (Bockaert and Pin, 1999).

During evolution, "molecular tinkering" of GPCR genes has been used to adapt their structure to recognize a wide range of environmental stimuli and regulatory molecules involved in cell–cell communication. The success of this adaptation was tremendous (Bockaert and Pin, 1999; Yokoyama *et al.,* 1999). GPCRs are implicated in recognizing messages as diverse as light (Shichida and Imai, 1998), Ca^{2+}, odorants of different chemical nature, amino acids, peptides, lipids, nucleotides, as well as proteins (Fig. 1A). They control the activity a diverse range of effectors such as enzymes (AC, phosphodiesterases, phospholipases, etc.), ionic channels (Ca^{2+}, K^{+}, Cl^{-}, etc.), transport vesicles, and kinases. They are also key elements in physiological functions, such as morphological movements during gastrulation and germ band extension, hormonal regulatory systems, synaptic transmission, and recognition of sensory stimuli (light, odorants, taste molecules, and pain stimuli). The odorant GPCRs are certainly the most abundant ones in most organisms, representing approximately 50% of the total number of GPCRs in *Drosophila* and vertebrates and as much as 90% in *C. elegans.*

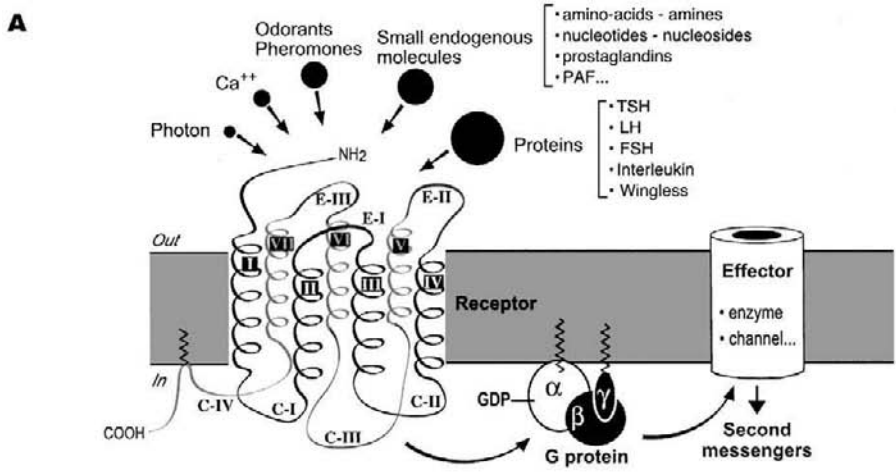
Such an important device for cell–cell communication in multicellular organisms has been a key element during their evolution. In addition, GPCRs are direct or indirect targets of the vast majority of available therapeutics drugs as well as drugs of abuse (Roush, 1996;Stadel *et al.,* 1997). Somatic and genetic mutations of GPCRs generate various pathologies (Birnbaumer, 1995; Spiegel, 1996; Van Sande *et al.,* 1995). Since several recent reviews are available (Bockaert and Pin, 1999; Gether, 2000; Wess, 1997), we summarize here most of the biochemical, pharmacological, and physiological knowledge of this old (almost 50 years) but still rapidly growing field.

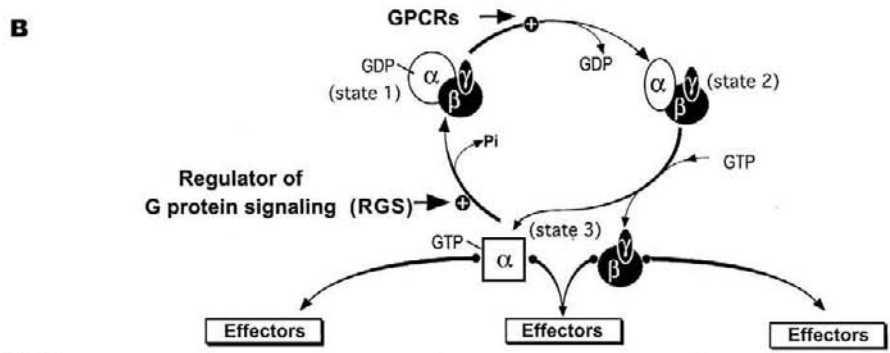
II. GPCR Signal Transduction

A. From a ``Ménage à Trois" to a ``Ménage à Quatre"

1. Rodbell's Model: Receptor, Transducer (G protein), and Effector

We previously mentioned the crucial experiments Rodbell performed on fat cells during the late 1960s (Birnbaumer and Rodbell, 1969). The nonadditivity of several hormonal receptors on AC activity posed a problem for Rodbell, who thought it inconceivable that several hormonal receptors could structurally anneal to the same enzyme (Rodbell, 1995). After long discussions with O. Hechter, Rodbell introduced the concept of hormone action as a ménage à trois. This concept, which is still valid today, was based on cybernetic theories of information introduced by Wiener (1961). The receptor was the equivalent of the discriminator, the AC was the amplifier, and the notion of transducer was introduced. The transducer was a coupling device designed to allow communication between the discriminator and the amplifier. At that time, the nature of the transducer was unknown and Rodbell





family							
α_s		Adenylyl-cyclases (all)	(+)	Adenylyl-cyclase (II, IV)	(+)	GIRK	(+)
				L type Ca^{++} channels	(+)	Phospholipase C (β_2)	(+)
α_i	α_t α-Gust	cGMP- phosphodiesterase	(+)			Ca^{++} channels (N/P)	(-)
	$\alpha_{i,\ 1,2,3}$	Adenylyl-cyclases (I, V, VI)	(-)			Adenylyl-cyclase (I)	(-)
	α_o	Adenylyl-cyclase (I)	(-)			Phospholipase A_2	(+)
		Ca^{++} channels	(-)			β-ARKinases	(+)
α_q	α_q, α_{11} $\alpha_{14\text{-}16}$	Phospholipases C (β_1 ,β_3)	(+)			MAP-Kinases	(+)
						PI_3-Kinases	(+)
α_{12}	α_{12}, α_{13}	Phospholipase A_2	(+)				
		Rac-cdc 42	(+)				
		Na^+/H^+ exchanger	(+)				
		Phospholipase C (ε)	(+)				
		Rho-GEFs	(+)				

FIG. 1 The "ménage à quatre" of GPCRs, (A) GPCRs are transmembrane proteins (TM-I–TM-VII) with three extracellular loops (E-I–E-III) and three cytoplasmic loops (C-I–C-III); a fourth loop after TM-VII is present in rhodopsin (see Fig. 2D) and probably also in many family 1 GPCRs. GPCRs have

had no idea that it could be a guanosine triphosphate (GTP) binding protein. This idea occurred a few months later, after a series of experiments, when the team observed that the binding of the hormone (the ^{125}I-glucagon) was acutely modified (increase in k_{on} and k_{off} and reduction of the "steady-state" binding level) by some components used in the AC assay. After a clever search for the "culprit," they found that it was GTP. They found not only that GTP modifies the hormonal binding characteristics but also that GTP is an absolute requirement to observe *in vitro* the stimulation of AC by receptors: The transducer was a GTP binding protein (G protein) (Rodbell, 1992).

The purification of the G protein by Gilman (Northup *et al.,* 1983) revealed the trimeric nature of G proteins. In their inactive form Fig. 1B, (state 1), the three subunits (Gα, Gβ, and Gγ) are associated and the Gα subunit binds GDP. The G$\beta\gamma$ complex is never dissociated under biological conditions and can be considered as a single unit. GPCRs can be considered as releasing exchange factors, functions homologous to those of monomeric small G proteins GDP-releasing factor (GRF) or guanine nucleotide exchange factor (GEF). Their role is to catalyze the GDP release from Gα. State 2 is then obtained (Fig. 1B) and is characterized as an empty nucleotide site. Gα and GPCR have different structures in state 2 than in states 1 and 3; they are tightly associated (Chabre and Deterre, 1989). The GPCR affinity for agonists is generally higher in state 2 than in states 1 and 3. It corresponds to the high agonist affinity state obtained in binding experiments when performed *in vitro* in the absence of GTP. State 2 is a short-life transition state. *In vivo,* GTP rapidly associates with the Gα empty state; dissociation then occurs. The Gα-GTP and G$\beta\gamma$ are split and dissociated from the activating GPCR Fig. 1B, (state 3). Gα and G$\beta\gamma$ can activate their respective effectors (Fig. 1B) (Bockaert and Pin, 1998; Clapham and Neer, 1993; Hamm, 1998). In some cases, both Gα and G$\beta\gamma$ are needed to activate their effector. This is the case for the activation of types II, IV, and presumably VII AC (Defer *et al.,* 2000; Tang and Gilman, 1991) and for some L-type Ca^{2+} channels (Fig. 1B). GTP hydrolysis is the only event that stops the activation of both Gα-GTP and G$\beta\gamma$. The reassociation of G$\beta\gamma$ with Gα-GDP also stops the action of G$\beta\gamma$ on the effectors. GTP hydrolysis is certainly tightly controlled, especially by proteins such as RGS (regulators of G-protein signaling) (De Vries *et al.,* 2000).

There are 17 genes (leading to at least 23 proteins) coding for Gα—5 for Gβ and 12 for Gγ (Hamm, 1998). In reality, not all the conceivable heterotrimeric G$\alpha\beta\gamma$ complexes are formed due to steric problems, especially for the GβGγ association (Lee *et al.,* 1995). The Gα proteins are divided into four families based on sequence similarities (Fig. 1B):

---

been tinkered with by evolution to recognize ligands such as photons, Ca^{2+}, amino acids, proteins, and lipids (Bockaert and Pin, 1999). (B) Activation cycle of heterotrimeric ($\alpha\beta\gamma$) G proteins by GPCRs. The GTPase activity of Gαs is activated by RGS (regulation of G protein signaling). The different families of G proteins and some of their effectors are listed.

-The Gαs family, which also comprises Gαolf (localized in olfactory neurons but also in striatum);
-The Gαi family, which comprises Gαt, the transducin implicated in visual transduction, and Gα-gus, expressed in gustative neurons but also Gαo and Gαz;
-The Gαq family, which also contains Gα11, Gα14, Gα15, and Gα16. Gα15 and Gα16 are homologous proteins expressed in hematopoietic cells of rodents and humans, respectively (Gomeza *et al.,* 1996b; Offermanns and Simon, 1995; Parmentier *et al.,* 1998). They have the remarkable property of being stimulated by GPCRs which are naturally coupled to Gq and also by those coupled to Gi/Go and even Gs;
-The Gα12, Gα13 family.

The crystal structures of Gα (Gαt and Gαi), as well as Gαβγ, have been obtained at a resolution of 2 or 3Å in an inactive form (GDP form) (Lambright *et al.,* 1994, 1996; Mixon *et al.,* 1995; Wall *et al.,* 1995) Fig. 1B, (state 1), under a transition mimicking state (GDP-AlF4-) either associated (Tesmer *et al.,* 1997a) or not (Sondek *et al.,* 1994) with RGS4; and in an active state (Gαs-GTPγS) either associated (Tesmer *et al.,* 1997b) or not (Coleman *et al.,* 1994; Noel *et al.,* 1993) with the catalytic domains of AC. Gα subunits contain two domains—a domain involved in binding and GTP hydrolysis (the G domain) which is structurally similar to the superfamily of GTPases, including small G proteins and elongation factors (the ras-like domain), and a unique helical domain (hα) that is inserted within the ras domain, which buries GTP in a pocket (Fig. 2; see color insert B). The Gβ subunits have a seven-membered β-propeller structure based on seven WD-40 repeats. Gγ subunits interact with Gβ through their N-terminal coiled coil and along Gβ. Gα subunits are myristoylated and/or palmitoylated at their N terminus, and Gγ is farnesylated or geranyl geranylated at its C terminal. These acyl and prenyl groups are the only devices controlling the association between the heterotrimeric Gαβγ complex and the membrane. Later, we discuss the mechanism by which GPCRs interact and activate G proteins. Reversible palmitoylation may play a regulatory role in GPCR action (Wedegaertner and Bourne, 1994). Long-term modulation of G protein mRNA levels has been reported (Milligan, 1993), but at protein levels G proteins are generally stable molecules with long lives (Brabet *et al.,* 1991).

2. New Partners in GPCR Activation of G proteins: The RGS

For many years, it has been known that Ras-type, monomeric, small G proteins have a very low GTPase activity. Specific proteins called GTPase activating proteins (GAPs) are required for the deactivation of these proteins (Boguski and McCormick, 1993). Reduction in the capacity of GAP to activate Ras GTPase activity is a common cause of malignancies. The mechanism of activation of Ras GTPase activity by GAP involves the coordination between a Gln (Q^{16} often mutated

in oncogenic Ras) and a Thr (T^{35}) of Ras, a glutamic acid residue of GAP, and a water molecule. The GAP puts its glutamic "finger" inside the Ras protein (Bourne, 1997a). Interestingly, in heterotrimeric G proteins, which have an intrinsic GTPase activity 1000-fold higher than that of the small G proteins, corresponding Gln (Q^{204} in Gαi and Q^{227} in Gαs) and Thr (T^{182} in Gαi and T^{204} in Gαs) coordinate a glutamic acid finger localized within the molecule (R^{178} in Gαi and R^{201} in Gαs localized in linker 2) and a water molecule to hydrolyze the GTP molecule. Mutations of the corresponding Gln and Arg in Gi or Gs have been found in tumors, and pathologies such as McCune–Albright and pseudo-hypoparathyroidism have been noted. In addition, ADP ribosylation of the R^{201} of Gs by cholera toxin reduces its GTPase activity, leaving the protein constitutively active. Overproduction of cAMP within the intestinal epithelial cells is responsible for diarrhea (Spiegel, 1996; Spiegel *et al.,* 1993).

For a long time, GTPase activity of purified heterotrimeric G proteins was recognized to be too slow to explain the deactivation of cellular processes under the regulation of G proteins. The $t_{\frac{1}{2}}$ of hydrolysis of GTP by most Gα proteins is in the 10- to 20-s range (Gilman, 1987) and even higher for Gαq (50 s) and Gαz (7 min) (Berstein *et al.,* 1992). The GTP hydrolysis on purified transducin (Gαt) is close to 15 s, although the deactivation of visual signals is close to 0.2 s (Navon and Fung, 1984; Vuong and Chabre, 1990). The Gαt effector, GMPc phosphodiesterase, has been proposed to be the GAP which accelerates GTP hydrolysis, but this is still a matter of controversy (Antonny *et al.,* 1993; Arshavsky and Bownds, 1992). The idea that the effector can serve as a GAP derived from the fact that the effector of Gαq, the phospholipase C β_1, is a GAP (Berstein *et al.,* 1992). The effector, particularly the phosphodiesterase (PDE) of rods, clearly acts in synergy with the real GAP of Gαt, the RGS9, both *in vitro* and *in vivo* (He *et al.,* 1998; Tsang *et al.,* 1998). The discovery of RGS occurred simultaneously from genetic studies and double-hybrid studies using Gαi3 as bait. In the latter, De Vries *et al.* were able to "fish" for a protein that they called GAIP (Gα interacting protein) (De Vries *et al.,* 1995, 2000; Dohlman and Thorner, 1997). In *C. elegans* and *Sacchromyces cerevisiae,* mutations within two genes—EGL-10 and SST2 respectively—were responsible for a delay in egg laying and reduced desensitization to pheromones, respectively (De Vries *et al.,* 2000; Dohlman and Thorner, 1997). In fact, the GAIP, EGL-10, and SST2 proteins were homologous within a 130-residue domain called the RGS domain (De Vries *et al.,* 2000; Wieland and Chen, 1999). This domain is responsible for binding to Gα proteins. RGS accelerate 50–100 times GTP hydrolysis of Gα proteins, which reduces the $t_{\frac{1}{2}}$ of GTP hydrolysis to a value compatible with the "physiology" (Berman *et al.,* 1996).

There are 30 mammalian RGS proteins (De Vries *et al.,* 2000), which differ considerably in size (17–160 kDa). Knowledge of the mechanism of action of RGS is still in its infancy. RGS is known to have a GAP function. This is confirmed *in vitro* but also *in vivo,* although mainly in recombinant systems. RGS clearly attenuates the action of GPCRs as shown with RGS4 and GAIP, which inhibit

the transduction of bradykinin (Gαq-mediated) and somatostatin (Gαi-mediated) receptors (De Vries *et al.,* 2000). In addition, binding of RGS to Gα results in inhibition of the effectors (Gα associations). RGS4 is able to inhibit GTPγS-activated phospholipase C (PLC)-β_1. Similarly, PLC-β_1 can displace RGS4 bound to Gαq-GDP-A1F4 (De Vries *et al.,* 2000). Finally, as previously discussed, the increase in Gα GTP hydrolysis, and therefore accumulation of Gα-GDP levels, accelerates the deactivation of GPCR transducing effects mediated via βγ. Overexpression of RGS1, RGS2, RGS4, and RGS8 accelerates the turning off of G protein-coupled inward rectifying K^+ channels (GIRKs), known to be activated by βγ (Doupnik *et al.,* 1997). Surprisingly, RGS also results in an increase in the k_{on} of GIRK activation by βγ (Doupnik *et al.,* 1997). Rod photoresponse recovery is slowed in mice lacking RGS9-1 (Chen *et al.,* 2000). RGS is made of nine α-helical structures that form two lobes—one made of helical structures α_1, α_2, α_3, α_8, and α_9 and the other made of helical structures α_4–α_7 (Tesmer *et al.,* 1997a). The base of α_3–α_4, α_5–α_6, and α_7–α_8 loops constitutes the contacting surface with the Gα subunit. The latter is made by the N-terminal domain of Gα, switches I and II, and to a lesser extent switch III (switch regions are the domains where there is a dramatic conformational change during the GTP cycle; see Fig. 2) (Sprang, 1997). Switch II is also part of the interacting surface of Gα with βγ and the effector AC as revealed in the cocrystal Gαs-GTPγS-AC (Tesmer *et al.,* 1997b). However, crystallographic data indicate that Gαi can accommodate RGS4 and AC at the same time (Sunahara *et al.,* 1997). Similarly, the synergism between the effect of RGS9 and PDEγ on the GTPase activity of Gαt suggests that both the effector and RGS can interact simultaneously with some Gα (He *et al.,* 1998). This is a priori contradictory with the idea that RGS can antagonize the Gα–effector association. The stabilization of the switch regions, particularly switch II (in which the important Arg residue implicated in GTP hydrolysis is localized), is certainly the basis of RGS action (Natochin *et al.,* 1998). Several residues of RGS4 (S^{85}, E^{87}, N^{88}, L^{159}, D^{163}, S^{164}, and R^{167}) encircle the Thr (T^{182} in Gαi1) engaged in GTP hydrolysis. N^{88}, L^{159}, and R^{167} seem to be the most important for the formation of the Thr^{182} pocket (Druey and Kehrl, 1997). However, more work has to be done to understand the structural basis of RGS mechanisms of action.

A major question is whether there is specificity in the RGS–Gα interaction. Most of the RGS proteins tested so far are GAPs for Gi/Go and Gq. p115Rho GEF (and perhaps PDZ–rhoGEF) (PDZ, a 90-residue domain first recognized in the postsynaptic density protein PSD95, in its *Drosophila* analog disc large tumor suppressor gene, and in the tight junction protein ZO-1) (Kornau *et al.,* 1997; Songyang *et al.,* 1997; Sudol, 1998) is a specific GAP for G12/G13. No RGSs have been found for Gαs (De Vries *et al.,* 2000), possibly due to the fact that the Asp (D^{229}) of Gαs (not conserved in the Gαi family) is localized in switch II and may constitute a barrier for its interaction with RGS. However, some subdomains of AC-V have been found to be able to accelerate both activation and deactivation of Gs (Siderovski *et al.,* 1999).

Most RGSs are present in the cytosolic pool, in plasma membrane (PM), and in intracellular membrane pools [especially membrane vesicles for Gα-interacting protein (GAIP)]. They can be recruited by Gα at the PM but can also have an intrinsic TM domain (RET-RGS1) and be palmitoylated (GAIP and RGS4). RGS12 has a PDZ domain which binds the C terminus of IL-8B and CXCR2 receptors (Snow *et al.,* 1998). RGS12 also binds and inhibits N-type Ca^{2+} channels (Schiff *et al.,* 2000). In addition to the RGS domain, RGSs contain structural domains at the N-terminal and the C-terminal positions. These structural domains connect RGSs to other interacting proteins (De Vries *et al.,* 2000).

In conclusion, the ménage à quatre in the GPCR transduction is well established. The implication of RGS in cancer, seizures, and drug tolerance is just starting to be investigated but is certainly promising (De Vries *et al.,* 2000). The regulation of RGS7 by tumor necrosis factor (TNF) and the endotoxin lipopolysaccharide is also interesting. Indeed, these proinflammatory and sleep-inclucing compounds inhibit the degradation of RGS7 by the proteasome system in the brain. This effect may certainly lead to a decrease in the Gαi/o-mediated antiinflammatory GPCR action and an increase in the secondary antiinflammatory effects of TNF mediated via Gs-associated GPCRs (Benzing *et al.,* 1999). Another intriguing finding is that newly discovered proteins, such as AGS (activator of G protein signaling), stimulate the GDP/GTP exchange on Gα proteins (Cismowski *et al.,* 2000). AGS1 is a ras-related protein possibly making a new link between small G and heterotrimeric G. Rapsynoid, a *Drosophila* protein, shares 43% identity with AGS (Parmentier *et al.,* 2000b). RGS14 has been proposed to be a Rap effector (Bidot *et al.,* 2000). As such, the ménage à quatre may already be old-fashioned.

B. Different Families of GPCRs and Their Binding Sites

Sequence comparison between the different GPCRs has revealed the existence of different receptor families sharing no sequence similarities (Bockaert and Pin, 1998, 1999; Josefsson, 1999; Kolakowski, 1994). However, all these receptors seem to share a central core domain made up of seven transmembrane domains (TM-I–TM-VII) which are helical tubes connected by three extracellular loops (E-I–E-III) and three cytoplasmic loops (C-I–C-III). Within the C terminus, a C-IV loop has been proposed to be formed between the TM-VII and the cysteine residues that are palmitoylated (Figs. 1 and 2). The crystal structure of rhodopsin revealed that this loop is an α-helical structure Fig. 2D (helice VIII). We constructed a phylogenic tree of 56 GPCRs taken from diverse eukaryotes, including yeast and slime mold (*Dictyostelium discoideum*), and the only GPCR cloned so far from the plant *Arabidopsis thaliana.* Protein sequences (excluding N-terminal and C-terminal domains) were multialigned and a tree was calculated using Clustal W. No further functional driven hypotheses were taken into consideration to adjust the alignment. Using this simple method we were able to classify these receptors

into eight distinct families (Fig. 3). Only a few receptors were not included in these families: the pheromone yeast GPCRs (STE2, STE3, and pheromone P), the *A. thaliana* putative GPCR (Josefsson and Rask, 1997; Plakidou-Dymock *et al.,* 1998), the Methuselah protein (an LNB-7TM protein of *Drosophila*) (Lin *et al.,* 1998), whose mutation extended life span and stress resistance, and the *Drosophila* putative taste receptors [gustatory receptor (GR) family Clyne *et al.,* 2000]. In addition, these receptors did not group together. Here, we do not describe the receptor for trehalose, recently cloned in *Drosophila,* which may represent a new subclass or family (Clyne *et al.,* 2000).

Bockaert and Pin (1998,1999) and others previously described six families of GPCRs using generally more polished alignments (e.g., without the intra- and extracellular loops). These families are also found in the present study: family 1, the rhodopsin receptor family; family 2, the parathyroid hormone (PTH), pituitary adenylate cyclase activating polypeptide (PACAP), and vasoactive intestinal peptide (VIP) receptor family; family 3, the glutamate metabotropic receptor (mGluR) family; family 4, one vomeronasal pheromone receptor family (VN); family 5, the frizzled receptor family; and family 6, the cAMP receptor family. There are two other families: family 7, the vertebrate T2R GR family which responds to bitter tastants (Adler *et al.,* 2000; Chandrashekar *et al.,* 2000) and family 8 which corresponds to a new family of putative odorant receptors in *Drosophila* (Clyne *et al.,* 1999; Gao and Chess, 1999; Vosshall *et al.,* 1999). Receptors from different families share no sequence similarity, suggesting that we are in the presence of a remarkable example of molecular convergence.

1. Family 1

It contains most of the GPCRs, including receptors for odorants (more than 1000). The crystal structure (2.8 Å) of rhodopsin was published recently (Fig. 2D) (Palczewski *et al.,* 2000). As expected from previous electron diffraction structures of rhodopsin (Unger *et al.,* 1997), TM-I, -IV, -VI, and -VII are bent at Pro residues, although in TM-I it is not significant and in TM-IV it is present at the extracellular end. Prolines are conserved in all members and play a crucial role in activation. TM-V, with a proline in the middle, is almost straight, whereas TM-II has a kink in the middle at the Gly–Gly doublet. TM-IV and -VII are shorter than the other TMs.

The following residues and sequences are considered to represent the signature of this family: First is an Asp (D) of TM-II which is essential for coupling to G proteins and which can form a hydrogen bond with the side chain of a conserved Asn (N) in TM-I and an Ala (A) in TM-VII in rhodopsin (Palczewski *et al.,* 2000). In other receptors, the Asp in TM-II may have a possible contact with an Asn in TM-VII. Complementing mutations in many receptors indicate for such a direct interaction. In any case, TM-I, TM-II, and TM-VII are in close contact (Fig. 2D) (Zhou *et al.,* 1994; Zhou and Fishman, 1991).

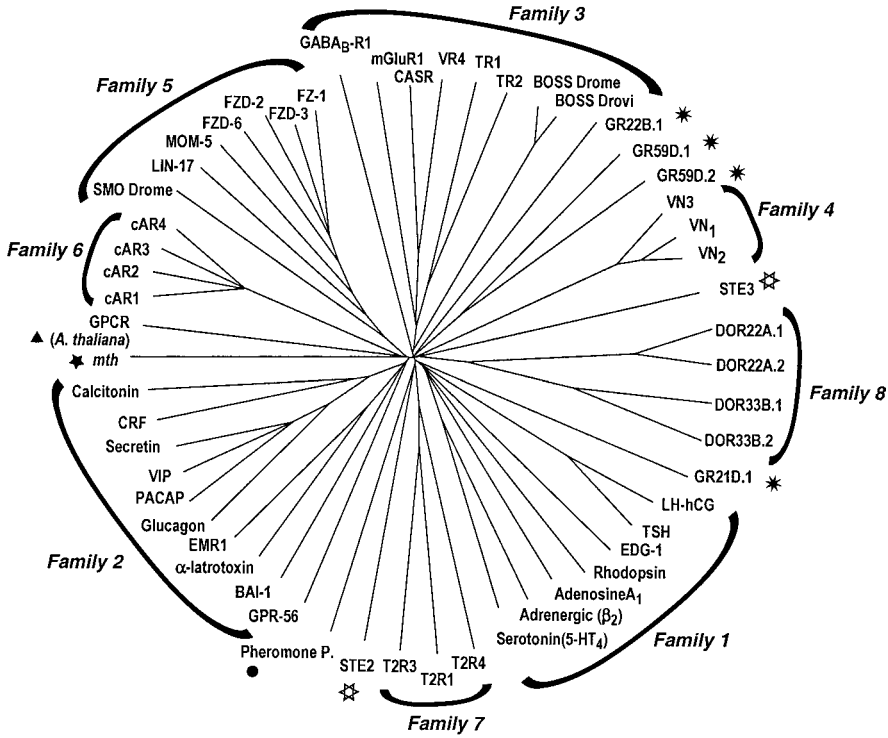


FIG. 3 The different GPCRs. Protein sequences (excluding N-terminal and C-terminal domains) were multialigned and a tree was calculated using ClustalW. A bootstrap analysis was performed on the tree construction; all branches with a bootstrap value lower than 80% were considered unreliable and were assigned a null length. The tree was drawn using TreeView (Page, 1996; Thompson *et al.,* 1997).

The accession numbers of GPCRs used to construct this tree are as follows: Family 1: 5-HT$_4$, P97288; adrenergic (β_2), Q28044; adenosine (A1), P47745; rhodopsin, P35362; EDG-1 (receptor for sphingosine-1 phosphate), M31210; TSH, P16473; LH-HCG, P22888; family 2: calcitonin-R, P79222; CRF-R (corticotropin-releasing factor 2), Q13324; secretin-R, 47872; VIP-R (vasoactive intestinal peptide), P32241; glucagon-R, P30082; EMR1 (cell surface glycoprotein F4/80), Q61549; α-latrotoxin-R, O88927; BAI-1 (brain-specific angiogenesis inhibitor), O14514; GPR56, AF106858; family 3: mGluR1, Q13255; VR4 (vertebrate putative pheromone receptor), O35192; TR1, TR2 (vertebrate putative taste receptor), Q9Z0R8; Boss–Drome (bride of sevenless protein of *Drosophila melanogaster*), P22815; Boss–Drovi (Boss protein of *Drosophila virilis*), Q24738; GABA$_B$-R1, O08620; CASR, P35384; family 4: VN1-3 (putative pheromone receptors), Q62850, Q62856, Q62852; family 5: FZ-1 (vertebrate frizzled receptors), Q08463; FZD2, FZD3, 6 (*Drosophila* frizzled receptors), Q94916, O00144, O60353; MOM-5 (*Caenorhabditis elegans* frizzled-like receptor), O16147; LIN-17 (*C. elegans* frizzled-like receptor), U63557; SMO-Drome (*Drosophila melanogaster* smoothened receptor), P91682; family 6: CAR1,2,3,4 (cAMP receptor of *Dictyostelium discoideum*), P13773, P34907, P35352, Q9TX43; family 7: T2R1,3,4 (bitter taste receptor), AAF43902, AAF43903, AAF43904; family 8: DOR (*Drosophila* odorant receptor), P81909, P81910, P81914, P81915, P81921. Extra family receptors: Yeast pheromone receptors: STE2, P06842; STE3, P06783; pheromone P, Q00619; putative receptors from *Arabidopsis thaliana,* O04214; Mth, Methuselah putative GPCRs, O97148; Putative *Drosophila* gustative receptors: GR59D1, ACO86245; GR59D2, AC006245; GR21D1, AC004420; GR22B1, AC003945.

Second, the Asp–Arg–Tyr (DRY), Glu–Arg–Tyr (ERY), or Glu–Arg–Tryp (ERW) sequences found at the N terminus of C-II are highly conserved and are directly involved in G protein activation (Oliviera *et al.,* 1994; Scheer *et al.,* 1996). Mutation of Arg in this sequence suppresses G protein activation. This sequence is surrounded by hydrophobic residues from H-II (Pro^{71} and Leu^{72} in rhodopsin), C-II (V136V137V138 and Phe^{148} in rhodopsin), H-V (Leu^{226} and Val^{230} in rhodopsin), and H-VI (Val^{250} and Met^{253} in rhodopsin). This region is likely to be a surface contact with G proteins (Palczewski *et al.,* 2000).

Third, basic residues at the end of C-III (Lys^{245}, Lys^{248}, and Arg^{252} in rhodopsin) are found at the position crucial for specific activation of G proteins (Bockaert and Pin, 1999; Bourne, 1997b).

Fourth, other intradomain constraints are likely important to keep the receptor under an inactive "R" state. This includes a bridge constraint made by Lys^{296} of TM-VII which is covalently attached (Schiff base) to the retinal. The retinal protonated Schiff base also makes a salt bridge with Glu^{113} in TM-III (Fig. 4). Mutation of Lys^{296} generates a constitutively active receptor and causes retinitis pigmentosa (Robinson *et al.,* 1992). In α_2-adrenergic receptors, this salt bridge is likely to occur between an Asp of TM-III and a Lys in TM-VII. Mutations of these residues resulted in constitutive activation of the receptors and complementing mutations reverse this phenotype (Porter and Perez, 1999). Interestingly, the binding of the protonated amine of adrenaline probably disturbs the salt bridge due to its binding to the Asp of TM-III. However, a Lys in TM-VII is not conserved in all biogenic amine receptors. Other constraints include hydrophobic interactions between a conserved Ile–Leu doublet of TM-III and a Phe of TM-VI (mutations of these residues constitutively activate the C5a receptors) (Baranski *et al.,* 1999) and a bridge between a highly conserved Tyr in TM-VII and Asn in TM-II (Palczewski *et al.,* 2000).

A cysteine–cysteine bridge is very well conserved between E-I and E-2 in all GPCRs of this family. In rhodopsin, the N-terminal domain contains two antiparallel β sheets that are located just below the E-II loop, which also contains two anti-parallel β sheets. All four β sheets, as well as most of E-II, make an extracellular plug blocking the exit from the pocket (Palczewski *et al.,* 2000). The E-II loop is almost inserted within the TM core, whereas the Asn^{2} is above the E-III loop in contact with Asp^{282}. The role of the plug in rhodopsin is not clear; however, it may prevent the all-trans retinal from projecting out of the pocket during activation. It remains to be determined if such plugs are located in other GPCRs of this family. Depending on the size and the location of the binding site, we proposed to divide this family into three subfamily groups (Bockaert and Pin, 1999).

Subfamily 1a contains GPCRs for small ligands, such as rhodopsin, catecholamines, odorants, small peptides, and ATP. The binding site of agonists is localized inside the seven TMs at a distance of about 10 Å (Palczewski *et al.,* 2000; Tota and Strader, 1990). Subfamily 1b includes most GPCRs for peptides in which the binding site is localized at the external face of the receptors, including the N-terminal,

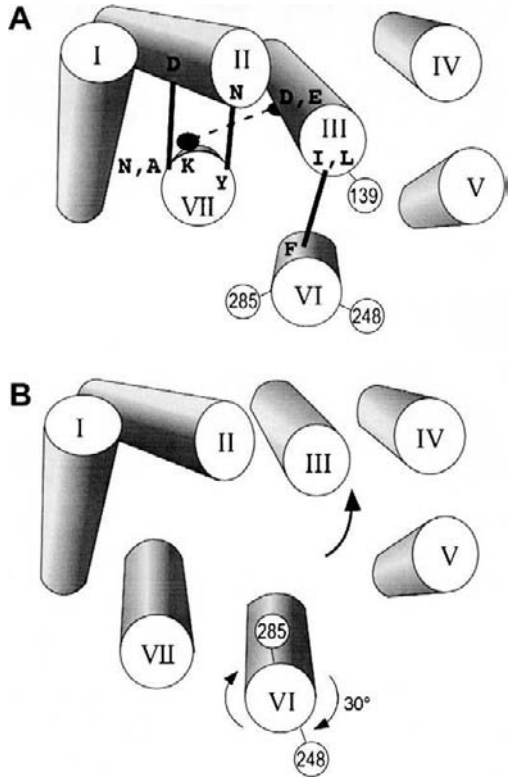


FIG. 4 The central core of family 1 GPCRs. (A) Inactive family 1 GPCRs (view from the cytoplasm). The TM included in the N- and C-terminal part of C-I and C-III which are considered to be α-helical structures (modified from Bourne, 1997b). The bars represent the structural constraint's between the α-helical structures. The dotted line represents the structural constraint's between TM-VII and TM-III provided by the retinal. For a description of the other constraint's, see Section III. (B) Activation of the central core of family 1 GPCRs (view from the cytoplasm). During activation, some of the constraint's are released. TM-VI rotates clockwise (30°). A cysteine introduced at position 285 moves from a hydrophobic domain to the hydrophilic cleft. The distance between TM-III and TM-VI increases. This opens a cleft in the central core in which the G proteins can find their way. For a further description, see Section III.

the extracellular loops, and the TM domains at the frontier of the extracellular surface (Bockaert and Pin, 1999). Family 1c includes GPCRs for glycoproteins, such as thyroid-stimulating hormone (TSH) and luteinizing hormone. The binding site is essentially extracellular, within the N-terminal domain, and made by a structure containing a series of leucine-rich repeats forming a crescent with the concave inner surface, consisting of β sheets which may bind ligands (Kajava *et al.,* 1995; Phang *et al.,* 1998).

The intracellular loops contain the classical three loops and, at least in rhodopsin but also likely in many other GPCRs, a fourth loop (C-IV) (Figs. 1A and 2D) made of an α-helical structure after TM-VII and before the cysteines of the C terminal which are palmitoylated. The corresponding peptides are reported to prevent rhodopsin from activating the G protein transducin (Gt) (Palczewski *et al.,* 2000). Such an α-helical structure in this C-IV region has been demonstrated in turkey β-adrenergic receptors by solution nuclear magnetic resonance spectroscopy (Jung *et al.,* 1996). This α-helical structure is amphiphilic, as is the C-terminal domain of C-III. Both regions are likely to be very important for the specificity and the potency of GPCRs to activate G proteins. C-II and C-IV project laterally from either side of the receptor, creating a platform of 43 Å that may be broad enough to allow rhodopsin to interact simultaneously with the α and γ subunits of G proteins (Bourne and Meng, 2000) (Fig. 2C). Several GPCRs from this family have particularly interesting structural or functional properties: Protease activated GPCRs (PARs) are activated when thrombin (PAR1, PAR3, and PAR4 receptors) or trypsin and tryptase (PAR2 and PAR4) bind to and cleave their amino-terminal exo-domain to unmask a new receptor amino terminus. This new amino terminus then serves as a tethered peptide agonist ligand, binding intramolecularly to the body of the receptor (Coughlin, 1999).

In many herpes and pox viruses, genes coding for homologs of chemokines, as well as chemokine GPCRs (Wells and Schwartz, 1997), have been described. The virus chemokine ligands are antagonists used to prevent the local recruitment of leukocytes. The function of virus GPCRs is less clear; some of them are constitutively active and may be implicated in malignancies such as Kaposi's sarcoma or acceleration of vascular diseases (Geras-Raaka *et al.,* 1998).

Entry of the human immunodeficiency virus (HIV) into human $CD4^+$ cells depends on the presence of both CD4 receptors and a GPCR that is generally CXCR4 (natural ligand, stromal derived factor-1) in T-tropic HIV-1 strains and CCR5 [natural ligands RANTES, macrophage inflammatory protein-1 alpha (MIP-1α), and MIP-1β] in M-tropic strains. T-tropic strains infect primary T lymphocytes and appear at the late stage of the disease, whereas M-tropic strains infect monocytes/macrophages and have been shown to be responsible for viral transmission (Berger *et al.,* 1999). The mechanism of HIV entry involves the interaction of the envelope protein gp160 (cleaved to gp120 and gp41 by the cell) with CD4, the association of gp120 with GPCR, and finally the dissociation of gp41 from gp120 and its participation in the virus fusion. A mutant allele of the CCR5 gene, Δ*32 ccr5,* found in Caucasian populations, was shown to provide homozygotes with a strong resistance to infection by HIV (Samson *et al.,* 1996). A north-to-south gradient was found with the highest allele frequency in Finland and Mordvinia (16%) and the lowest in Sardinia (4%). Data indicate that most *ccr5* alleles originate from a single mutation event that took place a few thousand years ago in northeastern Europe. The high frequency suggests that a selection advantage is associated with this mutation (Libert *et al.,* 1998). Note that the CCR5 variants account for a small proportion of individuals with apparent HIV resistance.

In addition, a structural variant of the chemokine receptor CX3CR1 appears to confer a more rapid progression of the pathology (Faure *et al.,* 2000). Drugs interfering with CCR5 and CXCR4 are actively researched and constitute a hope in the treatment of this infection.

2. Family 2

This family includes (Fig. 3), those for secretin, calcitonin, corticotropin-releasing factor (CRF), VIP, PACAP, and glucagon. The binding site is mostly extracellular, but E loops and part of the TMs exposed to extracellular domains are likely to be involved. Calcitonin and PACAP receptor N-terminal splice variants have been described to have a modified pharmacology (Houssami *et al.,* 1994; Pantaloni *et al.,* 1996). Note that we have included a series of receptors within this family (although Fig. 3 indicates that they are structurally closed but distinct from the classical list of hormonal receptors discussed previously) which belong to LNB-TM7. This is the case for the latrotoxin receptor, a receptor for the toxin of the black widow spider known to strongly stimulate neurotransmitter release in a Ca^{2+} -independent manner (Davletov *et al.,* 1998; Krasnoperov *et al.,* 1997). Also included in this subgroup is EMR1, a LNB-TM7 protein localized in macrophages, BAI-1, and also BAI-2 and BAI-3, which are LNB-TM7 proteins exclusively expressed in brain (Stacey *et al.,* 2000).

3. Family 3

The prototype of this family is the metabotropic glutamate receptors (mGluRs). The first one to be described was coupled to PLC (Nicoletti *et al.,* 1986; Sladeczek *et al.,* 1985). Eight mGluRs have been cloned and classified into three groups: group I (mGluR1 and -5), coupled to PLC; group II (mGluR2 and -3 and a *Drosophila* receptor DmGluRA; Parmentier *et al.,* 1996); and group III (mGluR4, -6, and -8). An N-terminal truncated mGluR is proposed to be a putative glutamate taste receptor (Chaudhari *et al.,* 2000) negatively coupled to adenylyl cyclase (Pin *et al.,* 1999; Pin and Duvoisin, 1995). The signaling properties of mGluRs are far more complex (Fagni *et al.,* 2000). Family 3 includes calcium-sensing receptors (CASRs), a specific family of pheromones (VR) (Bargmann, 1997), putative vertebrate taste receptors (Hoon *et al.,* 1999), $GABA_BR1$ (Kaupmann *et al.,* 1997) and $GABA_BR2$ receptors (Jones *et al.,* 1998a; Kaupmann *et al.,* 1998; Kuner *et al.,* 1999; Ng *et al.,* 1999; White *et al.,* 1998), and goldfish basic amino acids receptor (Speca *et al.,* 1999). Similar, but slightly distinct, is the bride of seven-less (BOSS), which is a ligand of seven-less tyrosine kinases involved in eye differentiation in *Drosophila* (Hart *et al.,* 1990). However, it has not been demonstrated that a G protein is required for its action.

Family 3 GPCRs possess a typical but unique feature: They have a large extracellular domain that shares some sequence similarities with bacterial periplasmic amino acid-binding proteins (PBPs), such as the leucine/isoleucine/valine-binding

protein (LIVBP) (O'Hara *et al.,* 1993) and acetamide-binding protein (Amic-C) (Bessis *et al.,* 2000). These proteins are characterized by a bilobate structure with ligand binding in a cleft which can open or close like a Venus flytrap. The extracellular domain of mGluR1 and -4 and $GABA_B1$ in isolation is enough to bind their ligands (Malitschek *et al.,* 1999; Okamoto *et al.,* 1998), a conclusion supported by the possibility of exchanging the extracellular domains of some of them, keeping the entire pharmacology associated (Parmentier *et al.,* 2000a; Pin *et al.,* 1999), and by the recent crystallization of extracellular domains of mGluR1 associated or not with glutamate (Kunishima *et al.,* 2000). Mutagenesis of both mGluRs (Pin *et al.,* 1999), $GABA_B1$ (Galvez *et al,* 2000a,b), and CASR as well as crystal structures of mG1uR1 (Kunishima *et al.,* 2000) and modelization of mG1uRs (Bessis *et al.,* 2000) and $GABA_BR$ (Galvez *et al.,* 2000a) give a clear picture of the binding sites.

Kunishima *et al.* (2000) determined the structure of two mG1uR1 unliganded forms and a complex form with glutamate (Fig. 5; see color insert.). All crystals contain two protomers (dimer) connected via a disulfide bridge between Cys^{140}. The dimerization of GPCRs is the subject of Section II.C. The N-terminal binding domain is made of two lobes, each comprised of two domains separated by three linkers (L1–L3) in the following order on the primary sequence and starting from the N terminus: LB1a, L1, LB2a, L2, LB1b, L3, and LB2b, followed by a cysteine-rich (CR) domain not present in $GABA_BRs$. The binding site is indeed localized within the cleft at the surface of lobe 1a (LB1a) in the open state and the bound ligand also contacts with LB2a in the closed state. Each domain can adopt an open or closed state; in addition, the two protomers in the dimer can adopt two positions called "active" (A) and "resting (R), differing in the orientation of an α-helical interface. The two unliganded crystallized forms correspond to a closed–open/A and an open–open/R form respectively, whereas the liganded form corresponds to only the closed–open/A form. There was no difference in the latter due to the presence of glutamate. This confirms previous hypotheses proposing that agonists stabilize rather than induce active forms. The feasibility of other putative conformers has been demonstrated, such as closed–open/R, closed–closed/R, open–open/A, and closed–closed/A (Kunishima *et al.,* 2000). In the open state, glutamate binds to two residues conserved in the entire mGluR family, including *Drosophila* and *C. elegans.* These residues are Ser^{165} and Thr^{188} in mGluR1, which form hydrogen bonds with the α-carboxy1 and the α-amino group, respectively. The γ-carboxyl is linked to a conserved Arg residue (Arg^{78} in mG1uR1) via a H_2O molecule and to Tyr^{74} via a hydrogen bond (Kunishima *et al.,* 2000). Additional contacts exist in the open as well as in the closed state (Kunishima *et al.,* 2000).

Mutagenesis and modeling of $GABA_BR1$ binding sites indicate that the carboxyl of GABA (equivalent of the γ-carboxy1 of glutamate) binds to Ser^{246} (homolog of Ser^{165} of mGluR1) in LB1a, whereas its amino group binds Asp^{471} in LB1b (Galvez *et al.,* 2000a). Ser^{269} (homolog of $Thre^{188}$ in mGluR1) is necessary for the potentiation of $GABA_BR1$ activation by Ca^{2+}, suggesting that Ca^{2+} can compensate for the lack of the α-amino group of GABA (Galvez *et al.,* 2000b).

4. Family 4

This is another family of putative pheromone receptors (VN1,-6) cloned from sensory Gαo neurons of the vomeronasal organ which coexpress the Gαi protein. This is in contrast to the observation that VRs from family 3 are expressed in vomeronasal neurons, which also express Gαo (Bargmann, 1997).

5. Family 5

The frizzled receptor family (11 members identified in mice) are either activated or inhibited by Wnt proteins (the name is derived from mouse Int-1 and *Drosophila* Wingless) and control embryonic development, cell proliferation and determination (Patapoutian and Reichardt, 2000; Perrimon, 1996). These receptors activate two downstream signaling pathways—one controls gene transcription and the other G protein-mediated Ca^{2+} influx. The immediate proteins activated by frizzled receptors are unknown, but the important adenomatous polyposis coli protein, often mutated in colorectal cancer, lies in its pathway. This family also contains receptors for "smooth" which are interesting because they appear to be under a permanent negative control of "patched," a 10-TM receptor for the morphogenetic factor "hedgehog." The binding of hedgehog to patched releases this inhibition (Perrimon, 1996; Ruiz i Altaba, 1997). MOM-5 and LIN-17 are *C. elegans* GPCRs; the latter are required for certain asymmetric cell divisions.

6. Family 6

This family of cAMP receptors has only been described in the slime mold *Dictyostelium discoideum* and is implicated in chemoattraction (Kim *et al.,* 1998; Klein *et al.,* 1988).

7. Family 7

This family comprises taste receptors (T2R) of vertebrates which mediate bitter taste via activation of gustducin, a G protein which activates cGMP phosphodiesterase and which is a homolog to transducin (Adler *et al.,* 2000; Chandrashekar *et al.,* 2000).

8. Family 8

This is the family for odorant *Drosophila* olfactory receptors localized in antenna and maxillary palp (Clyne *et al.,* 1999; Gao and Chess, 1999; Vosshall *et al.,* 1999).

To conclude this section on the diversity of GPCRs and to follow the idea that evolution has "tinkered" with GPCRs to adapt their structure to a wide range of ligands having very different structures (Bockaert and Pin, 1999), it can be noticed that genes coding for seven TM proteins may have fused with genes coding for

binding domains of other proteins to generate genes coding for new families of GPCRs. The same binding domains may also have fused with one TM protein to generate other receptors. Such a situation is found in glycoprotein hormone GPCRs in which the extracellular binding site is composed of several leucine repeats, similar to those found in Toll receptors (one TM receptor) implicated in inflammation (Hsu *et al.,* 1998; Kajava *et al.,* 1995). Secretin and frizzled receptors both share a CR extracellular amino terminus that is thought to be involved in ligand binding and which is conserved in diverse proteins, including several one-TM receptor tyrosine kinases such as those of the Ror family or the MusSK family (Xu and Nusse, 1998). Furthermore, as already discussed, the binding site of family 3 is homologous to the binding site of ionotropic glutamate receptors (Armstrong *et al.,* 1998), Ami-C (Bessis *et al.,* 2000), and atrial natriuretic peptide/guanylate cyclase receptors and prokaryote proteins such as PBPs (see *http://www.pharmsci.org/,* Vol. 1, issue 2).

C. Dimerization of GPCRs and Interaction with Other TM Proteins

For many years, the classical view of GPCR/G protein coupling stoichiometry was thought to be one receptor for one G protein. However, there was evidence in favor of the formation of dimers and even oligomers. Experimental evidence was provided in the early 1980s by several groups with the use of radiation inactivation, which indicated that GPCRs, G proteins, and AC acted as functional units that were larger than predicted for simple monomeric structures (Fraser and Venter, 1982; Salahpour *et al.,* 2000; Schlegel *et al.,* 1979). This was modeled by Rodbell in the 1990s (Rodbell, 1995). In addition, it was not conceivable that binding of ligands on the extracellular domain, like the one present in family 3 GPCRs, could modify the structure of the TM domains and intracellular loops without using dimerization or oligomerization in order to induce an allosteric change in the whole structure. This situation is similar to that demonstrated in tyrosine kinase receptors (Changeux and Edelstein, 1998).

1. Homodimerization of GPCRs

The recent interest in dimerization of GPCRs arose from functional studies in which inactive point-mutated (angiotensin AT1) (Monnot *et al.,* 1996) or chimeric receptors (α2-ARm3 muscarinic composed of the first five TM domains of one receptor and the last two TM domains of the other receptor) were not functional and did not bind ligands but recovered these properties when coexpressed (Maggio *et al.,* 1993). In the case of the chimeric receptors, both α2-AR binding and m3 binding as well as coupling were restored, which indicates that the interdimerization restored two "correct" binding pockets. Similarly, coexpression of CASRs that

harbor inactivating mutations in distinct domains also partially rescued Ca^{2+} responses (Bai *et al.,* 1999). In contrast, some mutated and truncated GPCRs have been shown to behave as dominant negatives. This is another indication of dimerization. In the case of truncated V2 vasopressin and CCR5 chemokine receptors, the dominant-negative effect was due to intracellular retention (Benkirane *et al.,* 1997; Vila-Coro *et al.,* 2000; Zhu and Wess, 1998). This may indicate that dimerization, at least for some GPCRs, is required for trafficking to the membrane. This role of dimers for trafficking to the membrane has also been demonstrated for the $GABA_B$ heterodimer (Margeta-Mitrovic *et al.,* 2000). In addition, the dominant-negative effect of CCR5Δ32 has been proposed to be the reason for the slow onset of the disease observed in AIDS patients who are heterozygous for this mutation (Benkirane *et al.,* 1997; Vila-Coro *et al.,* 2000).

In addition to functional studies, coimmunoprecipitation of differentially epitope-tagged receptors has allowed many studies to demonstrate the reality of the dimerization. Interestingly, dimerization has been demonstrated *in vivo* (*in cellulo*) using fluorescence resonance energy transfer in the case of alpha-mating factor receptor in living yeast (Overton and Blumer, 2000) or somatostatin receptors (Rocheville *et al.,* 2000b) and bioluminescence resonance energy transfer in the case of β2-AR (Angers *et al.,* 2000).

To date, dimerization has been found in the following receptors (this is not an exhaustive list):

Family 1 GPCRs: β2-adrenergic (Herbert *et al.,* 1996), angiotensin AT1 (Monnot *et al.,* 1996), δ-opioid (Cvejic and Devi, 1997), m3 muscarinic, somatostatin (Rocheville *et al.,* 2000b), vasopressin V2 (Schulz *et al.,* 2000), dopamine D1 (George *et al.,* 1998), and bradykinin B2 (Quitterer *et al.,* 1999)

Family 3 GPCRs: mGluR1 and mGluR 5 (Kunishima *et al.,* 2000; Okamoto *et al.,* 1998; Romano *et al.,* 1996; Tadokoro *et al.,* 1999; Tsuji *et al.,* 2000) and CASR (Bai *et al.,* 1998; Goldsmith *et al.,* 1999)

α-Mating factor GPCR (Overton and Blumer, 2000)

2. Heterodimerization of GPCRs

The first surprising observation that GPCRs can form heterodimers came from family 3. Cloning of $GABA_B$ receptors revealed that they are composed of two nonfunctional subunits ($GABA_BR1$ and $GABA_BR2$) sharing sequence similarity. Coexpression of $GABA_BR1$ and $GABA_BR2$ gave rise to a functional receptor, efficiently coupled to G proteins (Jones *et al.,* 1998a; Kaupmann *et al.,* 1998; Kuner *et al.,* 1999; White *et al.,* 1998). Recently, more surprise came from the observations that somatostatin and dopamine D2 GPCRs (Rocheville *et al.,* 2000a), κ and δ (Jordan and Devi, 1999) as well as μ and δ opioid receptors (George *et al.,* 2000), angiotensin AT1, and bradykinin B2 (AbdAlla *et al.,* 2000) can heterodimerize.

3. Structure of Homo- and Heterodimers

The diversity of solutions selected during evolution to form homo- and heterodimers is quite surprising, but it may indicate that formation of such dimers is crucial for generating diversity of coupling and pharmacology. Homodimerization via the N-terminal domain is found in the following receptors: bradykinin B2 receptors (AbdAlla *et al.,* 1999), mGluR1,5 (Kunishima *et al.,* 2000; Okamoto *et al.,* 1998; Romano *et al.,* 1996), and CASR (Goldsmith *et al.,* 1999). In the case of CASR and mGluRs, dimerization occurs via one conserved Cys residue localized on lobe I of the LIVBP binding site (Goldsmith *et al.,* 1999; Kunishima *et al.,* 2000; Romano *et al.,* 1996; Tsuji *et al.,* 2000). The S–S bond is not the only mechanism of dimerization (Robbins *et al.,* 1999). This bond certainly functions as an interpromoter linker to increase the concentration of dimers (Kunishima *et al.,* 2000). Dimerization has also been proposed to occur via the highly conserved Cys residues of E2, and E3 was found in m3 muscarinic receptors (Zeng and Wess, 1999).

Dimerization via TM regions has been suggested following observations that peptides corresponding to sequences present in these regions, especially those from TM-VI and TM-VII, disturb dimerization and sometimes functions of β_2-adrenergic (Hebert *et al.,* 1996), D2 dopaminergic (Ng *et al.,* 1996), vasopressin V2 (Schulz *et al.,* 2000), and CXCR4 receptors (Tarasova *et al.,* 1999). Homo- and heterodimerization via the C-terminal tail has been reported for δ-opioid receptors (Cvejic and Devi, 1997) and $GABA_BR1$ and $GABA_BR2$ receptors. In the latter, the interaction occurs via a coiled coil α-helical structure (Kammerer *et al.,* 1999; Kuner *et al.,* 1999).

4. Functional Roles of Homodimerization

The first question is whether agonists induce or increase dimerization. The answer is not clear: Agonists either do not modify dimerization (α-mating receptors; Overton and Blumer, 2000), slightly induce dimerization (β2-AR; Angers *et al.,* 2000), potently induce dimerization (bradykinin B2 receptors; AbdAlla *et al.,* 1999); somatostatin receptors; Rocheville *et al.,* 2000b) or even disrupt preexisting dimerization (δ-opioid receptors; Cvejic and Devi, 1997). The necessity for homodimerization for correct trafficking to the plasma membrane has already been discussed and is likely (Benkirane *et al.,* 1997; Vila-Coro *et al.,* 2000; Zhu and Wess, 1998). The necessity for homodimerization for coupling to G proteins has not been clearly demonstrated (Rocheville *et al.,* 2000b; however, see Cvejic and Devi, 1997).

5. Functional Roles of Heterodimerization

Before discussing the functional role of heterodimerization, evidence for such associations in naturally existing tissues has to be discussed imperatively. In the case of $GABA_B$ receptors, extensive colocalization of $GABA_BR1$ and $GABA_BR2$

subunits (except in striatum) favors such naturally occurring dimers (Kaupmann *et al.,* 1998). Similarly, somatostatin and dopamine D2 receptors are colocalized in brain cortex and striatum (Rocheville *et al.,* 2000a). An elegant experiment performed in smooth muscle cells indicates that antisense knockout of bradykinin B2 receptors inhibits the angiotensin AT1 response (AbdAlla *et al.,* 2000). The role of $GABA_BR1$ heterodimerization is quite clear. Alone, the $GABA_BR2$ subunit, which is inactive, is required for cell surface expression of the $GABA_BR1$ subunit (Jones *et al.,* 1998b; Kaupmann *et al.,* 1998; Kuner *et al.,* 1999; Margeta-Mitrovic *et al.,* 2000; White *et al.,* 1998). Indeed, $GABA_BR1$ is retained in the endoplasmic reticulum through a C-terminal retention/retrieval motif Arg–Lys–Arg (RKR), reminiscent of a similar sequence in ATP-sensitive K^+ channels. Interaction of $GABA_BR1$ and $GABA_BR2$ through their C-terminal coiled coil α-helical structure masks the retention signal in $GABA_BR1$ (Margeta-Mitrovic *et al.,* 2000). Moreover, Galvez *et al.* (2001) showed that the central core and intracellular domains of $GABA_BR2$ contain the molecular determinants required for recognition and activation of the G proteins. The central core and intracellular domains of $GABA_BR1$ improved coupling efficacy (Galvez *et al.,* 2001). In terms of pharmacology, heterodimerization may be a fine-tuning mechanism to increase the pharmacological diversity and coupling efficiency. In the AT1–bradykinin B2 dimer, the coupling of AT1 to messenger production is greatly increased by the presence of a functional or even a nonfunctional bradykinin B2 receptor (AbdAlla *et al.,* 2000). The pharmacology of the κ–δ opioid receptor dimers and μ–δ receptor dimers is obviously different from that of the corresponding homodimeric corresponding receptors (George *et al.,* 2000; Jordan and Devi, 1999). This is interesting for drug development, but at the same time it is quite puzzling.

6. Heterodimerization of GPCRs with Receptor Channels

The curiosity of some researchers regarding strange and, until recently, unbelievable associations led Liu *et al.* (2000) to search for a direct protein–protein coupling between dopamine D5 (a Gs-coupled GPCR) and $GABA_A$ ionotropic receptors. Indeed, both receptors are colocalized in dendritic shafts and the cell soma/axon hillock area where inhibitory GABAergic neurons form major postsynaptic contacts. These authors showed that the C terminus of D5 receptors (but not D1 receptors) selectively binds the second intracellular loop of the $GABA\gamma2$ receptor subunit. This physical association enables a mutually inhibitory functional interaction between these receptors. The physiological and therapeutic consequences of such an a priori bizarre marriage remain to be demonstrated.

7. Heterodimerization of GPCRs with One TM Protein

Nina A from *Drosophila melanogaster* and its vertebrate homolog RanBP2, two cyclophilin-related proteins, have been known for years to bind opsins for folding and transport (Baker *et al.,* 1994; Ferreira *et al.,* 1996). In *C. elegans,* odr-4 and

odr-8 are required to localize a subset of odorant GPCRs to cilia of olfactory neurons (Dwyer *et al.,* 1998). More surprising and disturbing for pharmacologists is the report that the calcitonin receptor-like receptor (CRLR) generates the (calcitonin gene-related peptide) receptor when associated with receptor activity-modifying protein (RAMP1) and the adrenomedullin receptor when associated with RAMP2. RAMP1 and -2 are also required for a correct glycosylation and transport of CRLR to plasma membranes. A yeast two-hybrid screen with the C-terminal 81 residues of the D1 receptor used as bait allowed Lezcano *et al.* (2000) to fish out a one-TM protein coding clone of the P19/21 calcyon family. Calcyon and dopamine D1 GPCRs are coexpressed in macaque brain within the spines of the CA3 region of hippocampus and caudate nucleus. Interestingly, D1 coupling to G_s is shifted to G_q when coexpressed with calcyon. In brain and kidney, D1 agonists have also be found to increase 1,4,5-trisphosphate turnover (Lezcano *et al.,* 2000).

III. Activities of GPCRs

A. Activation of GPCRs and G Proteins

The structures of the different families of GPCRs are so diverse that their mechanisms of activation are also likely to be quite different, although a common fundamental mechanism may exist at the final step.

1. Activation of the Central Core of GPCRs

The crucial observations that single mutations localized in intracellular loops, but also within the TMs or even in the extracellular loops or domains, may lead to constitutively active receptors is discussed in more detail in Section II.C. These data are in favor of an allosteric model in which constrained receptors R lose some intramolecular constraints and undergo a conversion to an active state R^*. We have already described some of these constraints (Fig. 4). In rhodopsin, a salt bridge exists between Lys^{296} of TM-VII (the retinal attachment site) and E^{113} in TM-III (the Schiff base counterion) (Palczewski *et al.,* 2000; Robinson *et al.,* 1992). There is also an interaction between Asn^{73} of TM-I and Tyr of the conserved motif NPXXY in TM-VII and interactions between Asn^{55} in TM-I, Asp^{83} in TM-II, and Ala^{299} in TM-VII as well as Asn^{302} (of NPXXY) via a water molecule (the so-called "polar pocket," also described in α1-AR; Scheer *et al.,* 1996) as described by Palczewski *et al.* (2000). This polar pocket is well conserved in family 1. In α1b-AR, a salt bridge between Asp^{125} of TM-III and Lys of TM-VII constrains the receptor in the R state (Porter and Perez, 1999). Similarly, in AT-1 receptor stabilization of TM-III and TM-VII, via Asn^{111} of TM-III and Tyr^{292} of TM-VII, has been described (Groblewski *et al.,* 1997). In C5a-R, many mutations of TM-III

and TM-VI lead to constitutive activation. Some of them suggest that hydrophobic constraints occur, for example, between Ile^{124}/Leu^{127} of TM-III and Phe^{251} in TM-VI (Fig. 4) (Baranski *et al.,* 1999).

Other constraints are evident in the rhodopsin crystal (Palczewski *et al.,* 2000) and it is likely that each receptor has its own. How these constraints are released and how agonists stabilize the active forms remain to be elucidated. One key event is certainly a change in the environmental situation of the DRY (or ERY) sequence and the resulting protonation of the aspartic/glutamic acid (Arnis *et al.,* 1994; Oliviera *et al.,* 1994; Scheer *et al.,* 1996,1997). This protonation has been directly measured in rhodopsin (Arnis *et al.,* 1994). An indirect argument in favor of this hypothesis is the fact that charged-neutralizing mutations which mimic the nonprotonated state of the aspartic acid cause dramatic constitutive activation of α1b, vasopressin, and rhodopsin GPCRs (Cohen *et al.,* 1993; Morin *et al.,* 1998; Scheer *et al.,* 1996). The role of arginine of the DRY sequence is crucial. Its mutation impairs the response mediated by GPCRs linked to different signaling pathways (Scheer *et al.,* 2000). Two hypotheses have been proposed to define the specific role of aspartate/arginine of DRY. In the first, arginine is constrained in the R state, in the polar pocket, causing an ionic interaction with the conserved aspartate in TM-II (Scheer *et al.,* 1996). Following activation and protonation of the aspartate of DRY, molecular modeling indicates that the arginine breaks its interaction with the aspartate of TM-II and shifts out of the pocket. In the second hypothesis, based on computational simulations in gonadotropin-releasing hormone (GnRH) receptors, it has been suggested that in the "inactive state" the ionic counterpart of the arginine of DRY is the adjacent aspartate (or glutamate) (Ballesteros *et al.,* 1998). Upon activation, this ionic interaction is broken and the arginine of DRY induces ionic interaction with the aspartate of TM-II. In favor of the latter hypothesis is the fact that mutation of the aspartate of TM-II disturbs functional coupling (Gether, 2000) and that in rhodopsin the aspartate of TM-II is more strongly hydrogen bound upon activation (Rath *et al.,* 1993).

The physical modifications of the central core of family 1 and 2 GPCRs are not understood, but some data have proven to be interesting. The main message is that the intramolecular constraints between the TMs are released. This is generally associated with thermal instability (Claeysen *et al.,* 2001; Gether *et al.,* 1997a; Rasmussen *et al.,* 1999) of the receptor and a separation of the TMs, leading to an increase in the crevice surface of the receptor forming contacts with G proteins (Fig. 4). Displacements have been observed between TM-III and TM-VI. Using the cysteine accessibility method, Javitch *et al.* (1997) noted that a cysteine in TM-VI (Cys^{285}), inaccessible to a hydrophilic sulfhydryl-reactive reagent in the R state, becomes accessible in constitutively active β2-AR. This indicates a clockwise rotation (looking at the receptor from the cytoplasmic surface) of TM-VI. Using tryptophan UV-absorbance spectroscopy, Lin and Sakmar (1996) obtained direct evidence that rhodopsin activation involves relative movements of TM-III and TM-VI. Site-directed labeling of single cysteines, inserted at the cytoplasmic side

of the transmembrane helical structures (Cys^{139} in TM-III and Cys^{285} and Cys^{248} in TM-VI) with sulfhydryl-specific nitroxide spin labels, provides evidence for clockwise (30°) and separation movements of TM-III and TM-VI (Farrens *et al.,* 1996). A recent cross-linking experiment indicates that the retinal ionone is attached to Trp^{265} in TM-VI, as predicted in the crystal structure in the R state (Borhan *et al.,* 2000). In the R^* state, the ionone ring is cross-linked to Ala^{169} of TM-IV. Bourne and Meng (2000) noted that if the *trans*-retinal interacts in such a way, this implies considerable movement of TM-III but also movement of TM-IV and VII. Similarly, in β2-AR, fluorescent labeling of cysteines in TM-III and TM-VI has provided a way of measuring an increase in fluorescence (accessibility to a more polar environment) upon agonist activation (Gether *et al.,* 1997b). Favoring the importance of the TM-III and TM-VI movements upon activation is the fact that binding of Zn^{2+} to the bis-His metal ion binding site, constructed between these TMs, blocks rhodopsin, β2-AR, and PTH receptor activation (Sheikh *et al.,* 1996, 1999). There is certainly some variability in the mechanisms by which the different families activate the central core. In family 1c, glycoprotein binding to the N-terminal part is likely to generate a special way of activation. G. Vassart's group (unpublished data) recently proposed that the N-terminal domain constrains the central core. This constraint would be released by the glycoprotein hormones. In the case of family 3, the twist between the two protomers of the dimer has been proposed to be associated with a relative movement of their central cores leading to activation.

In Section II.C, dealing with the dimerization of GPCRs, we clearly showed that homo- or heterodimerization of family 3 GPCRs is required for their activation. In family 1 GPCRs, the role of dimerization in activation is not quite as clear. The platform formed by the intracellular loops, including intracellular loop C-IV (43 Å), is sufficient for simultaneous interaction with the carboxyl terminal of Gα and Gγ (Bourne and Meng, 2000).

2. Intimate Contacts between GPCRs and G Proteins

Numerous studies have been dedicated to the research of GPCR domains that may confer specificity of interaction with particular G proteins. The general idea is that most intracellular domains, including the C-IV loop and the C-terminal domain, are implicated, whereas no consensus sequences have been recognized. It is possible that each receptor has designed its own solution. For two very close receptors such as vasopressin V2 (coupling to Gs) and V1a (coupling to Gq), the specificity of interaction is localized in C-I and C-III loops, respectively (Liu and Wess, 1996). In many family 1 GPCRs, the C-terminal part of the C-III loop determines the specificity. When four VTIL residues of the VTRTIL sequence, localized in the C terminal of C-III of muscarinic m_2 receptors (coupled to Gi), are introduced in the homologous positions of the muscarinic m3 receptor (coupled to Gq), the chimera is able to activate Gi (Liu *et al.,* 1995).

These four residues are likely to be on the same face of an α helix. There are six splice variants of PACAP receptors differing within the C-terminal part of the C-III loop by introduction of "hip" and "hop" cassettes. PACAP receptors can be coupled to both AC and PLC. The presence of the hip cassette suppresses the PLC coupling (Spengler *et al.,* 1993). Mutations of the key residue in the C-III intracellular loop often result in constitutive activation (Claeysen *et al.,* 2000; Kjelsberg *et al.,* 1992), In mGluRs, the C-II loop and the putative C-IV intracellular loop determine the specificity of coupling to G proteins (Gomeza *et al.,* 1996a,b; Mary *et al.,* 1998; Parmentier *et al.,* 1998; Prézeau *et al.,* 1996). Editing the 5-HT_{2C} mRNA within the sequence coding for the C-II loop leads to proteins with different coupling signaling characteristics (Burns *et al.,* 1997). Finally, phosphorylation of different intracellular domains induces a fine-tuning of the coupling to G proteins, including desensitization and change from Gs to Gi coupling (Daaka *et al.,* 1997). The capacity of mGluR5 to produce an oscillatory intracellular Ca^{2+} release depends on PKC phosphorylation of a specific threonine residue of the C-terminal domain (Kawabata *et al.,* 1996).

An important domain of Gα, implicated in the interaction and the specificity of a given GPCR to a given G protein, concerns the last few residues of their C terminus (Fig. 2). The residue -4 of Gαi, Gαo, and Gαt is a cysteine residue that is ADP ribosylated by pertussis toxin, a covalent modification that prevents the interaction of G proteins with receptors (Hamm, 1998). In the Gq family, residue -4 is a tyrosine that has to be phosphorylated for an efficient coupling to PLC-activating receptors (Umemori *et al.,* 1997). Gαq chimeras, in which the last four or five residues of Gq have been replaced by those of Gαi or Gαo, are still coupled to PLC, but they are activated by receptors naturally coupled to Gαi or Gαo, such as dopamine D2, muscarinic m2, and mGluR2, -3, -4, and -8. Interestingly, there are no sequence similarities between receptors from different families (Blahos *et al.,* 1998; Conklin *et al.,* 1993). The most important residue for the specificity of interaction is α4 in Gαi and Gαo, whereas two residues are important for the specificity of interaction with Gαq (Kostenis *et al.,* 1997). Two other domains of Gα are important for the coupling as well as for its specificity (Fig. 2)—the α4 and α5 helices—plus the L9 and β6, on the one hand (Mazzoni and Hamm, 1996; Noel *et al.,* 1993), and a short region between the N-terminal α helix and β_1 strand, on the other hand (Blahos *et al.,* 2001). A photoactivated peptide derived from the C-III loop of α2-AR is cross-linked with the N-terminal domain of Gαo and also with the C-terminal part of Gβ. Rhodopsin and β2-AR bind to Gβ (Taylor *et al.,* 1994, 1996). The scenario leading from these interactions to the change in the conformation of Gα, especially at the level of switches I–III, followed by the release of GTP (Fig. 2) is not known. Bourne (1997b) proposed two routes—one via α5–β6, a region in which a mutation induces rapid GDP release, and the other via a change in the orientation of Gα and Gβ due to the interaction of receptor intracellular loops, with the cavity separating these two subunits.

B. Orphan GPCRs

The current explosion of genomic sequence data provides a way to identify many more GPCR members in human and other species genomes. Most, if not all, newly identified GPCRs are in the category of orphan receptors for which the endogenous ligand remains to be identified. Typically, these GPCRs show only low levels of similarity (less than 35% sequence similarity) with known GPCRs. For family 1, more than 80 orphan GPCRs are known (Lee *et al.,* 2001).

The rationale for investing resources in characterizing orphan GPCRs is certainly to increase our fundamental knowledge, although this is no longer a pertinent driving force. A stronger motivation is the fact that GPCRs have a proven history of being excellent, directly or indirectly (e.g., antidepressant drugs), targets of the majority of drugs (Wilson *et al.,* 1998). Another reason is that there is an increasing number of diseases associated with GPCR mutations (see Section III.E). GPCR mutations and polymorphism are likely to be associated with pathologies and/or susceptibility to pathologies. The overall strategy has been called "reverse pharmacology." The research starts with extensive bioinformatical analysis of expressed sequence tags (ESTs) databases. ESTs are short sequences representing transcribed genes and continuous protein coding regions. They can be used to localize the GPCR tissue expression by *in situ* hybridization. Full-length clones can be obtained and chromosomal localizations determined. A possible association with genetic diseases can be analyzed. Once the full-length clone is obtained, the receptor can be expressed in heterologous cells and functional assays can be performed using a wide range of coupling mechanisms. The most popular assays are the measurements of changes in intracellular cAMP or Ca^{2+} levels either directly or through the use of reporter genes. The use of promiscuous G proteins, such as G15/G16 or chimeric Gqi, Gqo, and Gqz, is widely employed (Conklin *et al.,* 1993; Gomeza *et al.,* 1996b; Offermanns and Simon, 1995; Parmentier *et al.,* 1998). Once a functional assay is found, the search for the ligand can begin. Sometimes, a weak homology or a consensus sequence can be used to estimate whether the ligand for the orphan receptor is a peptide or a small ligand. Existing banks of compounds or biological extracts can be screened.

Every year, natural ligands for several orphan receptors are discovered (Lee *et al.,* 2001). The following ligands have been discovered:

Nociceptin (orphanin) (Meunier *et al.,* 1995; Reinscheid *et al.,* 1995)
Orexin (hypocretin) (Sakurai *et al.,* 1998)
Prolactin-releasing hormone (Hinuma *et al.,* 1998)
Apelin (Tatemoto *et al.,* 1998)
Melanin-concentrating hormone (Chambers *et al.,* 1999; Saito *et al.,* 1999)
Urotensin (Ames *et al.,* 1999)

We will not provide an exhaustive review of all six, but we will discuss two recent examples.

Prolactin secretion is very closely controlled by many neurotransmitters. Only dopamine inhibits prolactin secretion, whereas many others, such as TSH and VIP, can stimulate prolactin secretion. However, they were not thought to be the most important physiological stimulators of prolactin receptors. Hinuma *et al.* (1999) first cloned an orphan GPCR called hGR3, which was largely expressed in the pituitary gland. Expression of this GPCR in cells and stimulation with hypothalamus bovine extracts led to the release of arachidonic acid. The use of this functional assay in combination with chromatographic fractionation of the extracts led to the discovery of two peptides (PrP31 and PrP20) which stimulate the release of prolactin (Hinuma *et al.,* 1998). Hyperprolactinemia is a serious condition because this pathology inhibits reproduction. One can consider that mutations turning the hGR3 receptor to permanently active (see Section III.C), even in the absence of the PrP peptide, may be involved in the sterility of women suffering from hyperprolactinemia, especially those resistant to bromocryptine, a dopaminergic agonist which is the only drug available for this pathology. If this is the case, an inverse agonist may provide the solution.

The search for the ligand for an orphan receptor called HFGAN72, identified as an expressed sequence tag from human brain, was carried out in high-performance liquid chromatograph fractions of rat brain extract. The receptor was expressed in HEK cells and intracellular Ca^{2+} release was measured. This led to the discovery of two peptides termed "orexins A and B" after the Greek word *orexis,* which means appetite (Sakurai *et al.,* 1998). Both peptides are produced from a single precursor, the prepro-orexin. Precursor and orexia peptides are localized in neurons of the lateral and posterior hypothalamus. The projections of these neurons are directed to the olfactory bulb, cerebral cortex, thalamus, and the midbrain region, including the locus coeruleus, raphe nucleus, and reticular formation. When administered centrally to rats, these peptides stimulate food consumption. Additionally, the prepro-orexin mRNA level is upregulated upon fasting, suggesting a physiological role for the peptides as central feedback mechanisms that regulate feeding behavior. The role of orexin receptors in narcolepsy was recently described (Lin *et al.,* 1999).

Narcolepsy is a disabling sleep disorder affecting humans and animals; it is characterized by daytime sleepiness, cataplexy, and striking transitions from wakefulness into rapid eye movement sleep. Lin *et al.* (1999) determined that canine Doberman (canarc-1 dogs) narcolepsy is caused by disruption of the orexin receptor 2 gene (*Hcrtr2*). In parallel, Chemelli *et al.* (1999) reported that, by behavioral and electroencephalographic criteria, orexin knockout mice exhibit a phenotype strikingly similar to human narcolepsy patients as well as *canarc-1* mutant dogs. In addition, Modafinil, an antinarcoleptic drug with ill-defined mechanisms of action, was reported to activate orexin-containing neurons. Two recent reports indicate that in human narcolepsy, a reduction of orexin synthesis, rather than a mutation in its receptor, is involved (Peyron *et al.,* 2000; Thannickal *et al.,* 2000). Thus, the pathway to therapy is now clear. In 2 years, the orphan receptor has led to the discovery of peptides implicated in appetite and sleep physiology and

pathology. There is no doubt that orphan receptors will lead to new and exciting discoveries during the next few years.

C. Constitutive Activity of Allosteric GPCRs, Inverse Agonists, and Physiological Relevance

1. Drug–Receptor Models

Until the 1990s, pharmacologists working on GPCRs used the traditional "receptor occupation" theory that was the foundation of pharmacology for decades (Clark, 1933). The receptor was supposed to be "quiescent" under a R form in the absence of agonists (A). The agonist, when present, binds to R, inducing the formation of an AR complex ($A + R \leftrightarrows AR$) which triggers a cascade of events leading to physiological functions. The ternary complex model was introduced to account for the fact that GPCRs were found to bind agonists (not antagonists) with high and low affinity (De Lean *et al.,* 1980). This is because AR, but not R, is coupled with heterotrimeric G proteins and induces the GDP–GTP exchange (Fig. 1B). The ternary complex AR–G (state 2 in Fig. 1B, G protein empty) generally has a high affinity for the agonist, whereas AR and R generally have a lower affinity for the agonist. The antagonist has a unique affinity for R. The antagonist–R complex does not bind the G protein.

In 1965, Monod *et al.* wrote a famous article titled "On the Nature of Allosteric Transitions: A Plausible Model" (Changeux and Edelstein, 1998). The allosteric model proposed was based on observations made on bacterial enzymes that catalyze reactions of important biosynthetic pathways. They found that the catalytic activity of these enzymes can be regulated by the end product of the synthetic pathway which has no structural similarity to the substrate and acts on a specific regulatory binding site that is different from the catalytic site. The regulatory site induces a reversible alteration of the conformation of the protein—an allosteric transition. The observation that cooperative interactions exist for both the substrate and the ligand further extends the properties of allosteric proteins. The cooperativity of oxygen binding on the symmetrical tetramer hemes of hemoglobin separated by at least 25 Å further establishes the model. Several key features of allosteric proteins were first established for receptor channels, such as ionotropic receptors, nicotinic receptors, and GABA–benzodiazepine receptors (Changeux and Edelstein, 1998). First the protein is an oligomer composed of several subunits and posseses one axis of rotation. As noted previously, GPCRs are probably associated as a dimer and possibly as oligomers, although the exact role of dimerization is not always clear except for $GABA_B$ receptors and mGluRs (Galvez *et al.,* 2001; Kunishima *et al.,* 2000). Second, the oligomer can spontaneously exist in a minimum of two freely interconvertible and discrete conformational states. Third, the ligand binding stabilizes the particular state for which they exhibit a higher affinity. Finally, in the absence of agonists, the preexisting equilibrium between the inactive (R) and

the active (R^*) states is characterized by an isomerization constant *J.* Therefore, in the case of GPCRs we can write

$$R \overset{J}{\leftrightarrows} R^*, \text{ with } J = R/R^*$$

This isomerization step is the foundation for the extended ternary complex (Samama *et al.,* 1993). Indeed, this explains the increased affinity of agonists and partial agonists for the constitutively active mutant receptors, in proportion to their efficacy, even in the absence of G proteins (Samama *et al.,* 1993). In the ternary extended model, only R^* and R^*A can couple to G proteins. Several consequences were to be expected from such a model:

Depending on the value of *J*, GPCRs can exhibit constitutive activity (i.e., an activity in the absence of agonists) meaning that there is enough R^* coupled to G to activate a detectable amount of effector activity.

An antagonist is a drug which does not modify the equilibrium and therefore has an equal affinity for R and R^*,

An agonist is a drug with a higher affinity for R^* than for R and which therefore shifts the equilibrium toward the R^* form. The latter is the only one coupled to G proteins in the extended ternary complex model, which was first proposed by Lefkowitz and colleagues (1993).

Ligands having a higher affinity for R than for R^* should exist. In this case, they would displace the equilibrium toward R and reduce the constitutive activity, if any. These ligands are called inverse agonists.

Depending on the relative affinity for R and R^*, an agonist will be a full agonist or more or less partial.

Kenakin and Weiss (1996) introduced the cubic model, which differs from the ternary extended model by conveying the notion that R and R^* can couple to G (and of course RA and R^*A), even with different efficacies. Thermodynamically, this is the most complete model.

2. Inverse Agonists and Constitutively Active Receptors

The existence of inverse agonists was first proposed by Braestrup *et al.* (1982) when they reported that methyl 6,7-dimethoxy-4-ethyl-β-carboline-e-carboxylate (DMCM), in contrast to benzodiazepines, was a potent convulsant *in vivo.* DMCM seemed to favor binding on a closed conformational state of the $GABA_A$ channel (Braestrup *et al.,* 1982). Costa and Hertz (1989) were pioneers in demonstrating that some, but not all, δ-opioid receptor antagonists had a "negative" intrinsic activity in membranes prepared from NG108-15, i.e., they reduce basal adenylyl cyclase activity. Therefore, they proposed that the receptor could be under two states, only one in association with G proteins, in the absence of the ligand. In a pharmacological model, Wreggett and De Lean (1984) predicted that "antagonists"

may be active in hindering the ability of receptors to spontaneously associate with G proteins.

The existence of "inverse" agonists was a seminal observation that was pivotal for the existence of isomerization between R and R* (Chidiac *et al.,* 1994; de Ligt *et al.,* 2000). However, it was not until mutated GPCRs were discovered, which were able to adopt active conformations in the absence of ligands, and it was demonstrated that overexpression of native wild-type receptors generates enough active receptors to elicit a response that the concept was fully accepted.

The first description of a mutated constitutively active receptor was made by Lefkowitz and collaborators (Cotecchia *et al.,* 1990; Kjelsberg *et al.,* 1992). They reported that mutations of a key residue (A^{293}) in the C-III loop, a region critically involved in the activation of G proteins, are spontaneously active. The substitution of A^{293} for each of the other 19 amino acid residues led to receptors having a variable but always observable constitutive activity. This may indicate that in critical GPCR domains, evolution has selected the residue which allows constraint upon the receptor under the R form (Samama *et al.,* 1993). In certain human diseases, mutations of GPCRs are often the causal agents, as discussed later (Spiegel, 1996).

In theory, mutated receptors have a less constrained form and therefore a higher potency to isomerize from R to R*. This was found by measuring the isomerization *J* constant in COS cells for wild-type and constitutively active mutated 5-HT_4 receptors (Claeysen *et al.,* 2000). However, Claeysen *et al.* (2001) also found that mutated receptors have different R* structures. The existence of an equilibrium between an inactive form R and an active form R*, governed by the equilibrium constant *J,* also explains that overexpression of wild-type GPCRs, by virtue of increasing the absolute amount of R* (keeping the *J* constant unchanged), generates measurable constitutive activity both in cell lines and also *in vivo* (Chidiac *et al.,* 1994; Lefkowitz *et al.,* 1993). Bond *et al.* (1995) described transgenic mice overexpressing the wild β_2–AR at various receptor levels in atrial cells. Baseline left atrial tension in these transgenic mice was increased threefold over control mice, and ICI-118,551 acts as an inverse agonist, decreasing basal tension. The degree of ICI-118,551 inhibition was correlated with receptor density, suggesting that the event was receptor mediated.

In fact, some receptors are more or less constrained under the R form. In COS-7 cells, at a density of 300 fmol/mg (a density similar to that found in wild-type tissues), β_2-AR had no constitutive activity, whereas 5-HT_{4a}R increased cAMP production fourfold. Further increasing the density of β2-Ar generates constitutive activity (Claeysen *et al.,* 1999). In contrast, the wild-type PACAP receptor will not generate a constitutive activity event at high density (unpublished results from our laboratory).

Interestingly, splice variants of GPCRs and especially those showing splicing within the C-terminal domain have variable constitutive activities. There is increasing evidence that specific sequences of the C-terminal region of GPCRs modulate the isomerization between R and R* (Claeysen *et al.,* 1999; Joly *et al.,* 1995;

Prézeau *et al.,* 1996). A cluster of basic residues present in the C terminal of the short C-terminal forms of mGluR splice variants (mGluR1b, mGluR1c, and mGluR1d) impairs their ability to isomerize from R to R* (Mary *et al.,* 1998). Removing this cluster by mutation reveals their constitutive activity. In addition, the effect of this inhibitory cluster is suppressed by the long C-terminal domain of the other splice variant mGluR1a, which has a high constitutive activity (Mary *et al.,* 1998). Regarding the splice variants of 5-HT_4 receptors differing in the length of their C-terminal domain, the shorter the tail, the higher the constitutive activity. Artificial truncations revealed a domain of the tail, rich in Ser and Thr, which is largely engaged in constraining the receptor under the R form (Claeysen *et al.,* 1999).

Truncating or splicing out the last residues of thyrotropin-releasing hormone receptors and prostaglandin E receptors induces constitutive activity. The last 12 residues of bovine rhodopsin have also been proposed to be involved as negative regulators of the GTP exchange. Removing the C terminus of avian β-AR increases its activity (Hasegawa *et al.,* 1996; Matus-Leibovitch *et al.,* 1995; Parker and Ross, 1991; Weiss *et al.,* 1994). Either C-terminal-associated proteins or posttranslational modifications in the C-terminal domain are likely to be involved in the turning off of constitutive GPCR activity. This remains to be demonstrated. However, the existence of such interacting proteins, able to reduce the isomerization from R to R*, is possible. Their presence in tissues, in which these receptors are naturally expressed, and not in cell lines also explains why it is easy to obtain constitutive activity of GPCRs transfected in the latter. Recently, we found that the Homer proteins which interact with the C terminus of mGluR1,5 (see Fig. 7) are able to modulate the constitutive activity of these receptors (Ango *et al.,* 2001).

3. Constitutively Active Wild-Type Receptors in Native Tissues

Demonstrating the existence of constitutively active wild-type receptors in native tissues has in fact proved to be difficult (not considering cell lines which naturally express some GPCRs). There are several reasons for this difficulty. First, for reasons discussed previously, constitutive activity is low and undetectable in wild-type tissues. Second, to demonstrate such activity, one has to be sure that there are no agonists to contaminate the preparation. Finally, constitutive activity can only be demonstrated in native tissues if inverse agonists are available, which is only the case for some receptors. However, the list of GPCRs for which an inverse agonist is available is getting longer.

Inverse agonist effects have been observed in different cell lines without any need for overexpression of GPCRs (H.E.L. 92.1.7 cells endogeneously expressing the human α2-AR, RIN5AH cells expressing α2-D receptors and, as already discussed, NG108-15 cells expressing δ-opioid GPCR) (de Ligt *et al.,* 2000). In native tissues, there is convincing evidence that in myocytes, forskolin-stimulated Ca^{2+} currents can be inhibited by the β-adrenergic atenolol in the absence of agonists. Similarly, atropine seems to be able to block basal Ca^{2+} channel activity.

Bradykinin antagonists also seem to be able to decrease basal PLC activity in rat myometrial cells expressing endogenous receptors (de Ligt *et al.,* 2000). The overall demonstration of the existence of constitutively active GPCRs in native tissues is weak. However, Morisset *et al.* (2000) found that native histamine H3 receptors, which inhibit histamine release, are constitutively active. Physiologically, histamine H3 inverse agonists stimulated histamine release (Morisset *et al.,* 2000).

The idea that naturally existing inverse agonists can be found in some organisms is becoming more popular (de Ligt *et al.,* 2000). The agouti protein has been proposed to be an inverse agonist of melanocortin GPCRs, and exendin, a peptide isolated from the lizard, has been proposed to be an inverse agonist of glucagon-like peptide GPCRs. We already discussed the existence of constitutively active GPCR encoded within the genome of viruses such as Kaposi's sarcoma-associated herpesvirus, poxvirus, and cytomegalovirus. The herpes virus GPCR shows homology with vertebrate C–X–C receptors and is also referred to as ORF-74. An interferon-γ-inducible protein (IP10), agonist on C–X–C receptors, acts as an inverse agonist on ORF-74 (Geras-Raaka *et al.,* 1998). Other chemokines such as growth-related oncogenes are agonists, whereas stromal cell-derived Factor1a and viral macrophage inflammatory protein II (vMIP-II) are also inverse agonists of ORF-74 (Rosenkilde *et al.,* 1999). vMIP-II is also an antagonist of human chemokine GPCRs (Kledal *et al.,* 1997).

4. Effects of Long-Term Treatment with Inverse Agonists

The possibility of categorizing GPCR antagonists into two categories, neutral antagonists and inverse agonists, is not without consequences. Indeed, data show that chronic treatment with antagonists and inverse agonists does not result in similar long-term consequences. The classical effect of long-term treatment with inverse agonists is an upregulation of receptors that could be more pronounced than the upregulation observed with antagonists (MacEwan *et al.,* 1996; MacEwan and Milligan, 1996a,b). In both cases, stabilization of the occupied receptor occurs. Interestingly, long-term treatment with the 5-HT_{2C} inverse agonist SB206553 sensitized the PLC pathway both to 5-HT and to ATP (Berg *et al.,* 1999). An upregulation of Gαq/11 partially accounts for these observations. Similarly, Bouaboula *et al.* (1999) observed that treatment with a cannabinoid receptor (CB2) inverse agonist (SR 144528) also inhibits the lysophosphatidic acid receptor-mediated pathway, an effect attributed to the trapping of Gαi on CB2 occupied by SR 144528 (Bouaboula *et al.,*1999).

D. Diseases Caused by GPCR Mutations

In view of the physiological importance of GPCRs, it is not surprising that a series of pathologies have been found to be related to mutations of GPCRs (Spiegel, 1996). Most of these pathologies are related to obvious clinical manifestations,

such as blindness, diabetes insipidus, and hypo- or hyperthyroidism. Some undiscovered mutations, providing nonobvious phenotypes, are also likely to be responsible for pathologies such as psychiatric or neurological disorders. Some germline mutations may never be detected because they are incompatible with life. Both germline (inherited) and somatic (postzygotic) mutations have been found to be responsible for loss or gain of functions.

1. Loss-of-Function Mutations

These mutations are generally inherited disorders. Among them, the most common are certainly those that affect the gene coding for rhodopsin and vasopressin V2 receptors, which are responsible for retinitis pigmentosa (RP) (Dryja and Li, 1995) and nephrogenic diabetes insipidus (NDI), respectively (Arthus *et al.,* 2000; *www.medcon.mcgill.ca/nephros*). Approximately 100 and 117 mutant alleles have been identified as being causal agents in RP (autosomal-dominant except for three recessive alleles) and NDI (X-linked and autosomal-recessive) pathologies, respectively. In both cases, several types of receptor abnormalities have been found.

A minority of RPs exhibit no difference from wild-type receptors when studied in heterologous cells (Edwards *et al.,* 2000). In contrast, in photoreceptor cells, some of them (e.g., those affecting the last five residues of the C-terminal truncation or point mutation) appear to have a mislocalization in the photoreceptor cell (Edwards *et al.,* 2000). They remain in the cell body rather than in the membrane sacs within the rods (Dryja and Li, 1995). Indeed, as discussed later, the C terminus of rhodopsin interacts with TcTex-1, a dynein light-chain subunit (Tai *et al.,* 1999), allowing the transport of post-Golgi rhodopsin-containing vesicles along the microtubules toward the outer segment. The majority of mutations (most are missense mutations) lead to nonfunctional rhodopsin acting as dominant negatives. Indeed, haploinsufficiency is unlikely to be the problem since carriers of at least one apparent null allele are phenotypically normal (Rosenfeld *et al.,* 1992). In most cases, the protein is certainly misfolded and retained in the endoplasmic reticulum, causing photoreceptor degeneration.

In the majority of NDI mutants, the problem is the abnormal intracellular retention of the synthesized protein. The receptor is probably misfolded. Interestingly, permeant but not impermeant V2 vasopressin receptor antagonists have been found to dramatically increase cell surface expression and rescue function of eight mutant vasopressin receptors, thus acting as pharmacological chaperones (Morello *et al.,* 2000). This opens new therapeutic avenues for NDI and also for other GPCR-related pathologies due to misfolding of the protein. Another therapeutic possibility was proposed following the observation that cotransfection of mutated receptors (nonsense, frameshift, deletion, or missense mutations in the third intracellular loop or the last two TMs) with a C-terminus V2-R peptide spanning the sequence where the various mutations occur rescues functional receptors (Schoneberg *et al.,* 1996, 1997).

The diseases caused by loss of function of mutant GPCRs also include

Color blindness (cone opsins) (Nathans, 1999)
Familial ACTH resistance (Clark *et al.,* 1993; Naville *et al.,* 1996; Tsigos *et al.,* 1993)
Male pseudohermaphrodites undervirilization or female amenorrhea with low estrogen production (LH-R) (Misrahi *et al.,* 1998; Themmen *et al.,* 1997)
Male defect in spermatogenesis and female insensitivity to follicle-stimulating hormone (FSH) with ovarian dysfunction and infertility (FSH-R) (Misrahi *et al.,* 1998; Themmen *et al.,* 1997)
Familial or sporadic hypothyroidism (TSH-R) with phenotypes ranging from resistance to TSH to true hypothyroidism (Duprez *et al.,* 1999)
Familial hypocalciuric hypercalcaemia/neonatal severe primary hyperparathyroidism (CASR) (Brown, 1999)
Congenital bleeding (thromboxane A2-R) (Hirata *et al.,* 1994)
Hirschprung's disease (endothelin-R) (Puffenberger *et al.,* 1994)
Laron-type dwarfism (GH-R) (Godowski *et al.,* 1989; Okimura and Norton, 1998; Sanchez *et al.,* 1998; Wojcik *et al.,* 1998)
Familial GH deficiency (GHRH-R) (Maheshwari *et al.,* 1998; Salvatori *et al.,* 1999; Wajnrajch *et al.,* 1996)
Hypogonadism (GNRH-R) (Caron *et al.,* 1999; de Roux *et al.,* 1999a,b)
Blomstrand's chondrodysplasia (PTH/PTHr-R) (Zhang *et al.,* 1998)
Non-insulin-dependent diabetes mellitus (glucagon-R) (Hager *et al.,* 1995)
Obesity (melanocortin 4-R) (Hinney *et al.,* 1999; Vaisse *et al.,* 1998; Yeo *et al.,* 1998); narcolepsy in Doberman dogs (orexin-R) (Lin *et al.,* 1999)

2. Gain-of-Function Mutations

Site-directed mutagenesis of adrenergic receptors of the C-III loop by Lefkowitz's group led to the key notion that a mutated receptor can adopt an active R^* conformation in the absence of the ligand (Cotecchia *et al.,* 1992; Kjelsberg *et al.,* 1992). Constitutively active mutated receptors responsible for diseases were described soon after this discovery. One of them was the TSH receptor, for which a series of somatic mutations have been described in many patients suffering from autonomous thyroid adenomas (Duprez *et al.,* 1999; Parma *et al.,* 1993, 1995). The first described mutations were localized within the C-III loop and subsequent data indicate that a cluster of mutations is localized at the amino-terminal portion of TM-VI. However, other mutations are dispersed along the receptor in the extracellular domain, the extracellular loops, and TM-II, -III, -V, -VI, and -VII (Duprez *et al.,* 1999). Hereditary and sporadic toxic thyroid hyperplasia with growing goiter and the absence of autoimmunity, characterizing Graves disease, have been associated with mutations of TSH-R (Duprez *et al.,* 1999). Familial gestational hyperthyroidism can be observed in the absence of autoimmunity and normal

levels of human chorionic gonadotropin (hCG) A mutation affecting the extracellular domain of the TSH-R has been described. This mutation, which displays an increased sensitivity to stimulation by hCG, has the same response to TSH (Rodien *et al.,* 1998). The second receptor for which a constitutive activation has been described is LH-R, which causes familial male precocious puberty (Shenker *et al.,* 1993; Themmen *et al.,* 1997). Interestingly, there are no clinical manifestations in female carriers of the mutated gene. Spontaneous LH-R activation is sufficient to stimulate testosterone production and spermatogenesis, but it is inefficient in triggering puberty in females without concomitant FSH function.

The diseases caused by gain of functions of mutant GPCRs also include (i) congenital night blindness (rhodopsin) (Robinson *et al.,* 1992). The Lys^{296}–Met mutant in rhodopsin cannot bind 11-*cis*-retinal, the receptor is constitutively active, and this leads to retinal degeneration (Robinson *et al.,* 1992). This is supposed to be equivalent to constant light exposure, which is known to cause photoreceptor cell death; (ii) hypoparathyroidism (CASR) (Brown and Hebert, 1997); (iii) Jansen metaphyseal chondrodysplasia (PTH/PTHrP) (Schipani *et al.,* 1995, 1996); and (iv) pigmentation (somber and tobacco darkening) defect in mice (MSH-R) (Robbins *et al.,* 1993).

Genetic polymorphism of GPCRs frequently occurs (in contrast to rarer mutations) and probably underlies interindividual variability in both pharmacological responses and propensity to diseases: (i) A polymorphism in β_2-adrenergic-R (Arg^{16}–Gly) occurs more frequently in patients with nocturnal asthma, but its occurrence in hypertensive subjects is controversial (Buscher *et al.,* 1999; Strosberg, 1997); (ii) a polymorphism (Trp^{64}–Arg) of β_3-AR may be associated with earlier onset of non-insulin-dependent diabetes and morbid obesity; and (iii) the dopamine D4-R contains an unusual polymorphism composed of a 16-residue repeat region in the C-III loop. There are some pharmacological differences between the four- and the seven-repeat receptors. A group of subjects with the seven-repeat allele exhibited significantly elevated Novelty Seeking scores in comparison to subjects lacking the seven-repeat allele (Ebstein *et al.,* 1996). However, no link has been found between the seven-repeat allele and the deficit/hyperactivity disorder (Swanson *et al.,* 2000).

E. Allosteric Antagonists and Agonists of GPCRs

We call allosteric ligands those which do not bind to the binding site of the natural ligands. A long series of nonpeptidic antagonists for peptide GPCRs have been discovered due to high-throughput screening. They generally bind to the central core, composed of the seven TMs. Nonpeptidic antagonists for angiotensin (AT1 and AT2), bombesin, bradykinin, endothelin, opiates, vasopressin, oxytocin, neurotensin, substance P, and corticotropin-releasing factor, are now available (Betancur *et al.,* 1997). As opposed to peptidic antagonists, they have the

advantage of crossing the blood–brain barrier. In fact, for opiate GPCRs, allosteric agonists (morphine and heroin) and allosteric antagonists (naloxone) have been known for a long time. Nonpeptidic antagonists do not have the same binding site as peptidic agonists. For example, CP96345 inhibits the NK1 substance P receptor with a K_i of 14 n*M*. However, mutations localized in the extracellular regions of TM-V and TM-VI suppress CP96345 binding without modifying the binding of substance P (Gether *et al.,* 1993). The structural modifications of these regions are indeed crucial for receptor activation. Introducing histidines into these regions, followed by Zn^{2+} treatment, inhibits receptor activation but not substance P binding (Elling *et al.,* 1995). Steroid hormones may also possibly bind to the core domain of GPCRs acting as allosteric antagonists. This has been shown in the case of oxytocin-R, which is blocked by progesterone (Grazzini *et al.,* 1998). Aspirin and sodium salicylate allosterically inhibit endothelin receptors (Talbodec *et al.,* 2000). Interestingly, small and possibly allosteric antagonists of chemokine receptors, such as bicyclam derivatives for CXCR4 or UCB35625 for CXCR3, are likely compounds to be used in the future to block HIV infection (De Clercq, 2000; Sabroe *et al.,* 2000). Allosteric compounds acting on muscarinic receptors have also been described (Lazareno *et al.,* 2000).

In the case of family 3, high-affinity competitive antagonists have been particularly difficult to identify, probably due to the difficulty in modifying the structure of natural ligands while maintaining their affinity. Recently, novel, subtype-selective group I mGluR noncompetitive antagonists have been discovered which bind within the core seven-TM domain (which has not been involved in binding glutamate so far). CPCCOEt and BAY 36-7620 are specific mGluR1 noncompetitive antagonists, whereas MPEP is a mGluR5 noncompetitive antagonist (Carroll *et al.,* 2001; Litschig *et al.,* 1998; Pagano *et al.,* 2000). Synthetic molecules, such as NPS467 and NPS568, have been shown to potentiate the action of Ca^{2+} on CASR (Hammerland *et al.,* 1999; Nemeth *et al.,* 1998). Alone, neither compound has an agonist effects, but together they induce a shift to the left of the Ca^{2+} concentration response curves, indicating that they act as allosteric modulators. Ca^{2+} alone may be a positive allosteric ligand acting at a site close to the binding site of glutamate in mGluRs (Kubo *et al.,* 1998) and GABA in $GABA_B$ receptors (Galvez *et al.,* 2000b).

F. Posttranscriptional and Posttranslational Modifications of GPCRs

It has been noted that GPCR–G protein interactions are complex and diverse. In addition to splicing with insertions localized mainly at the C-III loop and the C-terminal domains, GPCR domains implicated in G protein interactions are susceptible to fine-tuning via posttranscriptional and posttranslational modifications.

1. RNA Editing

Transcripts encoding the 5-HT_{2C} receptor, a PLC- and phospholipase A2-coupled receptor, undergo RNA editing events in which the genomically encoded adenosine residues are converted to inosines by double-stranded RNA adenosine deaminase(s). Seven major 5-HT_{2C}-R isoforms are predicted, encoded by 11 distinct RNA species, differing in their second intracellular loops. These modifications lead to changes in the efficacy of coupling to G proteins. Agonist stimulation of the nonedited human receptor (5-HT_{2C}-INI) and the edited 5-HT_{2C}-VSV and 5-HT_{2C}-VGV receptor variants stably expressed in NIH-3T3 fibroblasts demonstrated that serotoninergic agonists were less potent at the edited receptors (Burns *et al.,* 1997). This reduced G protein coupling for the edited isoforms is primarily due to silencing of the constitutive activity of the nonedited 5-HT_{2C}-R.

2. Phosphorylation Associated with Desensitization

Several mechanisms regulate the length and strength of GPCR signals. For a series of recent reviews, see Bohm *et al.* (1997), Bunemann and Hosey (1999), Bunemann *et al.* (1999), Pitcher *et al.* (1998), and Tsao and Zastrow (2000). Generally, desensitization refers to a progressive loss of the physiological response and the effector activity, despite the continued presence of the signaling ligand. Two types of desensitization have been described. Homologous desensitization refers to the situation in which only the activated GPCRs desensitize, whereas heterologous desensitization refers to the situation in which activation of one GPCR leads to the desensitization of responses initiated by another heterologous GPCR. There are multiple mechanisms of receptor desensitization, but they are not all understood. The most rapid phase of desensitization (minutes) involves agonist-induced phosphorylation either by classical second messenger-activated kinases (mainly in heterologous desensitization) or by GPCR-activated kinases, followed by binding to adapter proteins such as arrestins. Sequestration or internalization is a second event that commonly occurs with a slightly slower time course. The receptor can be recycled at the cell surface or destroyed in lysosomes (downregulation).

In heterologous desensitization, classical second messenger-activated protein kinases are implicated. β_2-ARs are excellent substrates for cAMP protein kinase (PKA), and their phosphorylation on the C-III loop leads to an uncoupling from Gs. Recent reports suggest that PKA-mediated phosphorylation can increase β_2-adrenergic receptor coupling to Gi and promote MAP kinase cascade activation (Daaka *et al.,* 1997; Luttrell *et al.,* 1999). However, this may not be a general mechanism (Luttrell *et al.,* 1999; Tsao and Zastrow, 2000). Protein kinase C (PKC)-mediated heterologous desensitization has also been reported (Bunemann *et al.,* 1999). A complex between the A-kinase-anchoring protein, AKAP79/150, PKA, and PKC facilitates receptor phosphorylation. Homologous desensitization appears to involve phosphorylation by a unique family of GRKs (Pitcher *et al.,*

1998). Six GRKs have been identified: GRK1 (rhodopsin-kinase localized in the retina), GRK2 and -3 (β-adrenergic receptor kinases, β-ARK1 and -2), and GRK4, -5, and -6. GRK4 is localized in testis, and the others have a wide distribution.

The mechanisms of association of GRKs to the plasma membrane are diverse. GRK1 is isoprenylated, whereas GRK4 and -6 are palmitoylated. GRK2 and -3 are translocated to the membrane when $\beta\gamma$ is released from G proteins upon activation by the phospholipid environment. Phosphorylation by GRKs occurs either in the C-terminus domain [(β_2-AR, rhodopsin, and somatostatin (SSTR3)) or in the C-III loop (α_2-AR and muscarinic-R (m2)]. GRKs are subject to regulation by PKA, PKC, GRK, and Ca^{2+}-binding proteins such as recoverin (for GRK1) and calmodulin (Pitcher *et al.,* 1998). Phosphorylation does not necessarily mean uncoupling. After GRK-induced phosphorylation of rhodopsin and β_2-AR, uncoupling occurs when arrestins (four arrestins have been identified; arrestin-1 is found only in the retina) bind to the phosphorylated receptors. Other uncoupling or adapter proteins may exist. The classical pathway for β_2-AR endocytosis involves its targeting to coated pits, although it can also be targeted, as are some other GPCRs, to caveolae (Tsao and Zastrow, 2000). The targeting to coated pits is associated with the binding of several proteins to arrestin, such as the AP-2 adapter, NSF (*N*-ethylmaleimide-sensitive fusion protein), clathrin, and Src (Laporte *et al.,* 1999, 2000; McDonald *et al.,* 1999; Tsao and Zastrow, 2000). For some, but not all, muscarinic receptors, dynamin, a GTPase that forms the necks of the vesicles, is required in coated pits and for caveolae-mediated endocytosis (Ahn *et al.,* 1999; Bunemann *et al.,* 1999). Internalization of Src via GPCR-induced endocytosis has been proposed to be the mechanism by which these receptors activate the MAP kinase cascade. This hypothesis has been questioned by other researchers.

After being internalized in endosomes, the receptors can be recycled following dephosphorylation by a specific member of phosphatase 2A and 2B families. Recycling of β_2-AR requires interaction of its PDZ C-terminus ligand binding domain with the EBP_{50}/NEHRF erzin–actin complex (see Section III.F) (Cao *et al.,* 1999). Alternatively, the receptor (vasopressin V2) can be sequestered for a long time in an unknown intracellular compartment (Innamorati *et al.,* 1999; Oakley *et al.,* 1999) or degraded in lysosomes (downregulation) or in nonlysosomal compartments (Tsao and Zastrow, 2000). Whether the pathways involved in endocytosis and downregulation are always similar or not remains to be clarified. Downregulation of β_2-AR also involves a decrease in receptor synthesis and destabilization of mRNA (Bohm *et al.,* 1997). Divergent residues located at the C terminus of thrombin and substance P receptors specify differences in trafficking between lysosomal and recycling pathways.

The role of GRKs has also been studied *in vivo* using transgenic mice. GRKs in GRK2 knockout mice were found to be lethal to the embryo at the age of 15 days. They had severe defects in cardiac development and most likely died of cardiac failure. Mice overexpressing GRK2 showed an increased cardiac function (Pitcher *et al.,* 1998). GRK3 (β-arrestin2) determines morphine tolerance but not dependence (Bohn *et al.,* 2000).

IV. Interactions of GPCRs with Other Proteins

A. GPCRs as Unfaithful Receptors to G Proteins

It is not surprising that GPCRs interact with many other proteins in addition to G proteins (Hall *et al.,* 1999). This has already been discussed in the section on desensitization, where GPCRs have also been shown to interact with arrestins. This is a consequence of receptor activation via G proteins and will not be discussed here. More surprising is that GPCRs also directly interact with a wide variety of proteins which are important for some of their functions (clustering, anchoring, optimization of transduction, transduction not mediated through G proteins, etc.) and which do not implicate G proteins.

1. Association with PDZ Domain-Containing Proteins

Various proteins have been recognized to contain PDZ domains, 90-residue domains first recognized in PSD-95/SAP90, its *Drosophila* homolog. Three groups of PDZ ligands have been recognized: Group I PDZ binds to C-terminal peptides with the consensus E–(S/T)–X–Φ (in most cases, Φ is V or I); group II binds peptides with the Φ–X–Φ triplet; and group III binds peptides with a less defined consensus sequence such as $\varphi\varphi\Phi$, as exemplified in β-neurexine YYV (Φ is a hydrophobic residue and φ is an aromatic residue) (Daniels *et al.,* 1998; Maximov *et al.,* 1999; Sudol, 1998). However, some variations on this theme are likely. Many ionic channels, receptor channels, enzymes, and synaptic protein C termini contain a PDZ binding sequence which interacts with the PDZ of multidomain proteins. They form vast complexes in the synapse (Ehlers *et al.,* 1998; O'Brien *et al.,* 1998).

Many GPCR C termini also contain PDZ binding ligand (Figs. 6 and 7). β_2-AR associates via a C-terminal PDZ binding domain with the Na^+/H^+ exchanger regulator factor (NEHRF) and its homolog, the erzin–radixin–moesin binding phosphoprotein-50 (EBP-50) (Fig. 6A) (Cao *et al.,* 1999; Hall *et al.,* 1998a,b). This protein contains two PDZs; the first one interacts with the DSLL C-terminus sequence of β_2-AR. The interaction of β_2-AR and NEHRF has solved a mystery in the β_2-AR signaling pathway involved in the regulation of the Na^+/H^+ transporter. Indeed, for a long time, β_2-AR was thought to be coupled only to Gs and the only second messenger implied was cAMP. However, deletion of parts of the C-III intracellular loop of β_2-AR suppressed the Gs coupling as expected but activation of the Na^+/H^+ exchange by β_2-AR agonists was maintained (Barber *et al.,* 1992). This was a clear indication of the existence of another signaling pathway not requiring Gs and the C-III loop. Hall *et al.* (1998a), using a β_2-AR tail-GST protein, fished out NEHRF. In the absence of β_2-AR agonists, NEHRF binds to the Na^+/H^+ exchanger and inhibits its activity (Fig. 6A). NEHRF associated with activated β_2-AR suppresses the inhibition of NEHRF, leading to stimulation of the

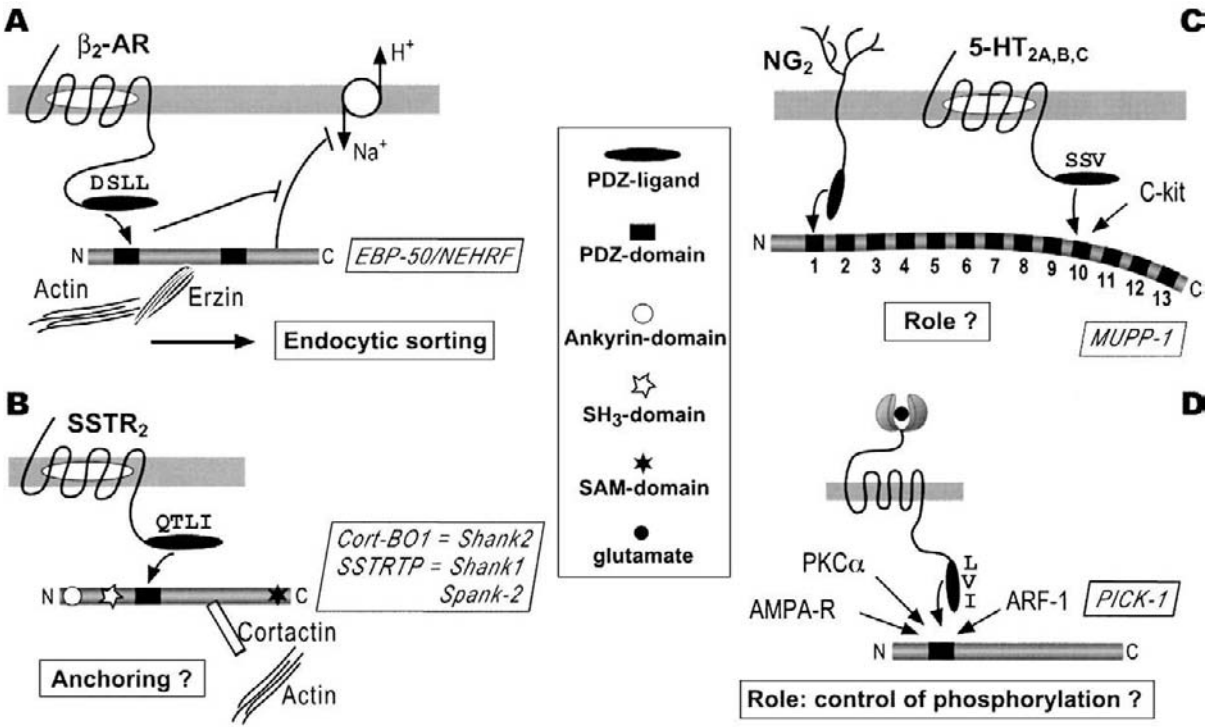


FIG. 6 GPCR interactions with PDZ proteins. For further information, see Section IV.

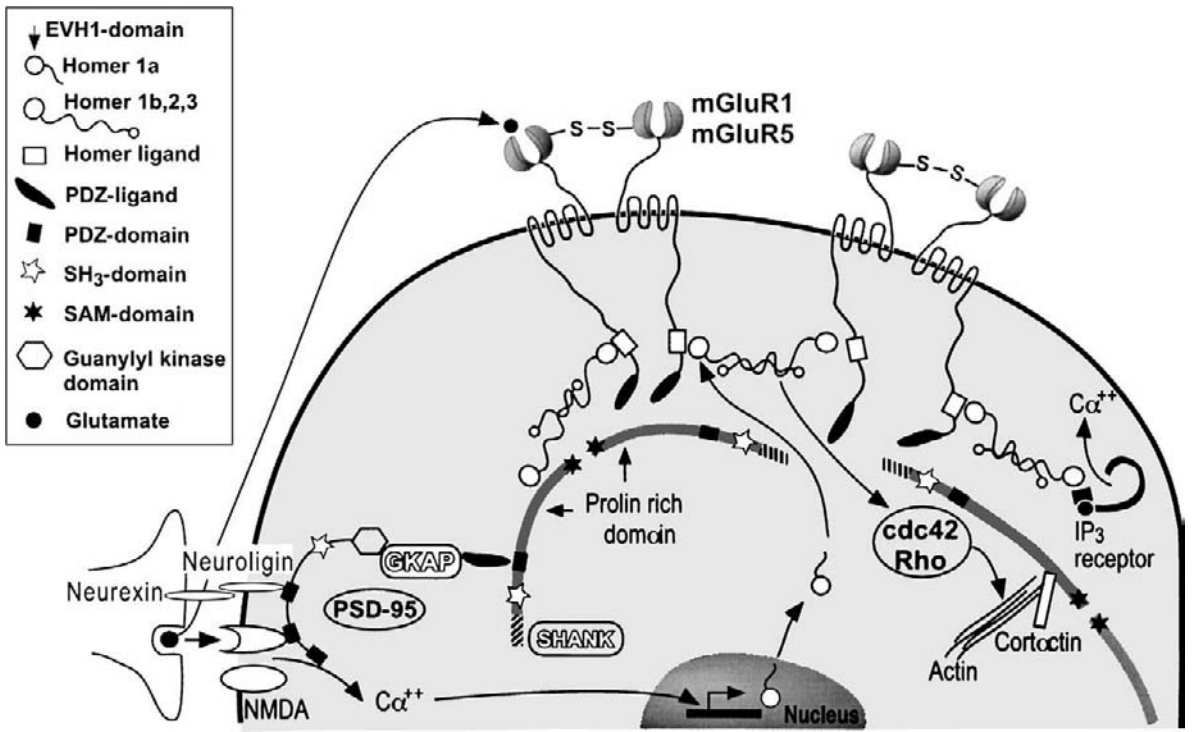


FIG. 7 The glutamate postsynaptic synapse. For a complete description, see Section IV.

Na^+/H^+ exchanger (Fig. 6A). Mutation experiments indicate that the PDZ binding domain of β_2-AR is involved. P2Y1-R and the cystic fibrosis TM regulator also interact via their PDZ binding domains with the NEHRF protein (Hall *et al.,* 1998b). In addition to the regulation of the Na^+/H^+ exchange, the β_2-AR–EBP-50 erzin–action complex controls the recycling of the receptor after endocytosis. Phosphorylation of the serine (Ser^{411}) of the DSLL binding domain by GRK-5 inhibits the association of β_2-AR with EBP-50/NEHRF and receptor recycling (Cao *et al.,* 1999).

Somatostatin acts through five different GPCRs (SSTR1–R5). SSTR2 has been shown to interact via its QTLI–PDZ binding domain with two related proteins, cortactin-binding protein 1 (CortBP1) and SSTRIP (Fig. 6B) (Zitzer *et al.,* 1999a,b). In fact, CortBP1 and SSTRIP multidomain proteins originate from the same family. CortBP1 is also called Shank2 and is homologous to ProSAP1, whereas SSTRIP is homologous to Shank1, Spank1, and synamon (Fig. 7). These proteins contian various domains, such as an ankyrine domain at the N terminus, an SH3 domain, a PDZ domain, a proline-rich domain to which cortactin binds, and a sterile a-motif domain at its C terminus which is implicated in dimerization (Fig. 7). SSTR2 associated with CortBP1 is stimulated by somatostatin. SSTR2 associates with actin via cortactin, an interaction implicated in its anchoring in nerve terminals, in which it regulates neurotransmitter release via the inhibition of N-terminal calcium channels.

Using the two-hybrid screen, in which the C terminus of the 5-HT_{2C} receptor was a bait, MUPP1 (multi-PDZ-domain protein), a 13-PDZ domain protein, was isolated (Ullmer *et al.,* 1998). Protein alignment of all PDZ domains from INADL (inactivating no after-potential D-like), C52A11.4, and MUPP1 revealed that all three proteins share extremely identical PDZ domains. Moreover, the most similar PDZ domains are arrayed in the same order resulting in the same organization. 5-HT_{2C}, 5-HT_{2B}, and 5-HT_{2A} receptors interact with PDZ 10 (Fig. 6C) via their C-terminus SSV, SYV, and SCV, respectively. The MUPP1–5-HT_{2C} interaction was demonstrated in transfected COS cells and also in choroid plexus. Coclustering and change in the conformation of MUPP1 occur during the interaction (Bécamel *et al.,* 2001). MUPP1 also interacts with NG2 via PDZ1 and with c-Kit via its PDZ 10 (Barritt *et al.,* 2000; Mancini *et al.,* 2000). The discovery of additional proteins that interact with other PDZ domains of MUPP1 should reveal its real function. Note that resensitization and coupling of 5-HT_2-R to NO-S have been reported to implicate the C-terminus PDZ ligand of 5-HT_2-R. For these 5-HT_2-R-mediated transduction processes, the interacting PDZ protein has been identified (Backstrom *et al.,* 2000; Manivet *et al.,* 2000).

The N-terminus PDZ domain of RGS12 has been shown to interact with a PDZ-binding sequence of the A/S–T–X–(L/V)-type domain. The search for the possible interacting proteins indicated that the C terminus of the interleukin-8 receptor B (CXCR2) and that of the alternative 3′ exon form of RGS12 are likely partners (Snow *et al.,* 1998).

mGluR7a receptors interact with the protein-interacting C kinase (PICK1), a 1-PDZ binding protein, via their C terminus (LVI). A much larger C-terminal domain is needed for tight binding. The PICK1 PDZ site also interacts with AMPA receptors, ephrin ligands and receptors, and class I ADP ribosylation factors. Since PICK1 can dimerize, a clustering of these molecules is likely. PICK1 interacts with the C-terminal of PKC-α (QSAV) and modulates the phosphorylation of mGluR7a by PKC-α (Dev *et al.,* 2000).

The *Drosophila* phototransduction cascade is not a cGMP phosphodiesterase-mediated pathway but a PLC-β pathway leading activation of store-operated Ca^{2+} entry channels [transient receptor potential calcium channel (TRP)] and their associated nonspecific cationic conductance channel TRPL (light-activated channels) (Montell, 1997). PLC-β is activated by the rhodopsin–Gq complex (Tsunoda *et al.,* 1997; Xu *et al.,* 1998). A remarkable characteristic of this cascade is that rhodopsin, Gαq, PLC-β, TRP, TRPL, and PKC all bind (directly or indirectly) with INAD, a 5-PDZ domain-containing protein) (Bahner *et al.,* 2000; Montell, 1997). TRP and TRPL clearly interact via their C-terminal PDZ binding domains; however, the mode of interaction of other proteins in the cascade is not as obvious. If one notes that INAD forms homomultimers, then a vast complex is clearly involved (transduciosomes). INAD also interacts with calmodulin and an unconventional myosin, NINAC (Montell, 1997).

2. Association with EVH1 Domain-Containing Proteins

A family of proteins which appear to function as a link between cell surface signals (including focal adhesion proteins, zyxin and vinculin, or the axon guidance receptors SAX-3/Robo) and actin-based cytoskeleton has recently been described. These proteins include *Drosophila*-enabled (Ena, termed Mena in mouse), yeast Bee1p, vasodilator-stimulated phosphoprotein (VASP), and the Wiscott–Aldrich syndrome protein (WASp), and these encode one EVH1 domain which binds the consensus proline-rich motif FPPPP (Prehoda *et al.,* 1999).

The Homer protein family belongs to the VASP/WASp superfamily but contains another type of EVH1 domain with a slightly different structure. This domain, which binds the consensus sequence (PPXXF), has already been described (Beneken *et al.,* 2000). The first member, called Homer1a, Ves1-1S, and Ania-3 (named respectively by the three groups involved in its discovery), was identified on the basis of the rapid upregulation of its mRNA (immediate early gene) following seizures, long-term potentiation, and cocaine and dopaminergic D1 stimulation (Berke *et al.,* 1998; Brakeman *et al.,* 1997; Kato *et al.,* 1997). Homer1a can be considered as an immediate early gene. The N terminus (110 residues) essentially contains the EVH1 domain, which has been shown to interact with group I mGluRs, IP3, and ryanodine receptors as well as Shank (CortBP1/ProSAP1/spank/SSTRIP) proteins (Fig. 7) (Naisbitt *et al.,* 1999; Tu *et al.,* 1998, 1999; Xiao *et al.,* 1998). The other members of the Homer family contain an additional C terminus with

a predicted coiled coil (CC) structure, including leucine zippers which allow them to dimerize or multimerize. These members comprise (i) two other Homer1 splice variants, differing by 12 residues [Homer1b and Homer1c (= PSD-Zip45 = Ves1-1L)]; (ii) two Homer2 splice variants, differing by 11 residues [Homer2a (= cupidin, = Ves1-2D11) and Homer2b (= Vesl-2)]; and (iii) one Homer3. One *Drosophila* Homer (D-Homer) has also been cloned, as have other splice variants (Fagni *et al.,* 2000; Kato *et al.,* 1998; Shiraishi *et al.,* 1999; Tadokoro *et al.,* 1999; Xiao *et al.,* 1998, 2000).

The PPSPF domain of mGluR5, approximately 50 residues from the C terminus (Fig. 7), is essential for binding the EVH1 domain of Homers, whereas its C-terminus PDZ ligand may interact with the PDZ domain of Shank (Naisbitt *et al.,* 1999; Xiao *et al.,* 2000). Because NMDA receptors also interact with this Shank PDZ domain via the PSD-95–GKAP (guanylate kinase associated protein) heterodimer and Shank dimerizes and also interacts with Cortactin and therefore actin (Fig. 7), this constitutes a colossal postsynaptic complex. The Homer dimers, or multimers, may directly connect mGluR group I to IP3 and ryanodine receptors, shown to be engaged in their signaling pathways. Induction of Homer1a, which does not dimerize (because it does not contain the CC domain), following active neuronal activation has been shown to modify the mGluR1-induced kinetic of intracellular Ca^{2+} release (Tu *et al.,* 1998). A role of Homers in cellular trafficking has also been reported (Xiao *et al.,* 2000). In cerebellar neurons, Homer1b and Homer1a are required to address a stabilized mGluR5 to dendrites as well as dendrites plus axons, respectively (Ango *et al.,* 2000). Homer proteins also control the constitutive activation of group I mGluR5 (Ango *et al.,* 2001).

3. Association with Enzymes and Transcription Factors

Several GPCRs, especially angiotensin AT1 and 5-HT_{2A}, have been shown to activate the Jak kinases, the first element in the cytokine receptor signaling cascade. These two receptors have been shown to bind Jak kinase in an agonist-dependent manner (Guillet-Deniau *et al.,* 1997; Marrero *et al.,* 1995). In AT1 receptors, the critical binding motif (YIPP) is localized within the proximal part of the C terminus (319–322) (Ali *et al.,* 1997). Tyrosine phosphorylation of this motif by Src is necessary for binding the SH2 domain of SHP-2 (tyrosine phosphatase), which then binds Jak/STAT. The same phosphorylated tyrosine is necessary for the binding of PLC-γ1 on the same YIPP motif (Venema *et al.,* 1998). Interaction of CXCR4 receptors and Jak2/Jak3 kinases has also been reported (Vila-Coro *et al.,* 1999).

Another interesting case is the association of bradykinin B2 receptor with endothelium and neuronal nitric oxide synthases (eNOS and nNOS) (Golser *et al.,* 2000; Ju *et al.,* 1998). eNOS forms an inhibitory complex with bradykinin receptors likely via the blockade of the flavin to heme electron transfer. The inhibitory complex is released in a ligand- and Ca^{2+}-dependent manner due to tyrosine phosphorylation of the eNOS interacting region of the receptor. A similar interaction of

angiotensin AT1 and endothelin-1 ETB receptors with eNOS has been described (Marrero *et al.,* 1999). In all three receptors, the receptor-interacting domain is localized within the C-IV region (Ju *et al.,* 1998).

Interestingly, the C termini of $GABA_BR1$ and -R2, which contribute to their heterodimerization, are engaged in an interaction with the leucine zipper domain of ATF-4 (CREB2), a transcription factor of the CREB/ATF family. ATF-4 and $GABA_B$ receptors are colocalized within soma and dendrites of cultured hippocampal neurons. Nehring *et al.* (2000) and White *et al.* (2000) demonstrated that $GABA_B$ receptors activate ATF-4 translocation to the nucleus and gene transcription in heterologous systems.

4. Association with Arrestins and Associated Proteins

We already noted that arrestins bind to phosphorylated GPCRs. This event is the first step in endocytosis. Arrestins recruit several other proteins, such as c-Src, NSF, AP2, and clathrin. This complex and the Src-induced phosphorylating dynamin, within the coated pits, lead to the formation of endocytotic vesicles. Endocytosis of Src, leading to the activation of the MAP kinase cascade mediated by GPCR, has been proposed (see Section IV.B and Fig. 8).

5. Miscellaneous

Interestingly, the C-IV region is also a binding site for Ca^{2+}/calmodulin in mGluR7 (O'Connor *et al.,* 1999). This binding appears to be competitive with $G\beta\gamma$ and is blocked following PKC phosphorylation of serine residues within the same region (Nakajima *et al.,* 1999). O'Connor *et al.* proposed a mechanism by which Ca^{2+}/calmodulin is required to release $G\beta\gamma$ from the C terminus of mGluR7 in order to obtain a $G\beta\gamma$-mediated N-type Ca^{2+} channel. Similarly, two regions of mGluR5 (and mGluR1a but not mGluR1b,c) interact with Ca^{2+}/calmodulin (Minakami *et al.,* 1997). These regions are localized within the C terminus (842–869 and 922–950) in mGluR5. A domain 10 residues upstream from the last C-terminal domain (339–348) of the angiotensin AT1 receptor interacts with another protein, ATRAP (AT1 receptor-associated protein) (Daviet *et al.,* 1999). Functional studies suggest that this protein inhibits AT1 coupling to PLC.

The C terminus of rhodopsin (Gln^{344}–VAPA) is highly conserved among vertebrates and is a hot spot for mutations that cause some forms of RP. Two of these C-termini mutants (Gln^{344}–ter, characterized by the removing the last five residues, and Pro^{347}–Leu) are characterized by a specific defect in the transport of rhodopsin (Sung *et al.,* 1994; Tai *et al.,* 1999). Mice that express Gln^{344}–ter rhodopsin show abnormal rhodopsin accumulation in the plasma membrane (instead of the outer segment discs) and cell body, where it is synthesized (Sung *et al.,* 1994). This shows that the C terminus is required for efficient transportation to, or retention in, the outer segment. Recently, the C terminus has been shown to interact with

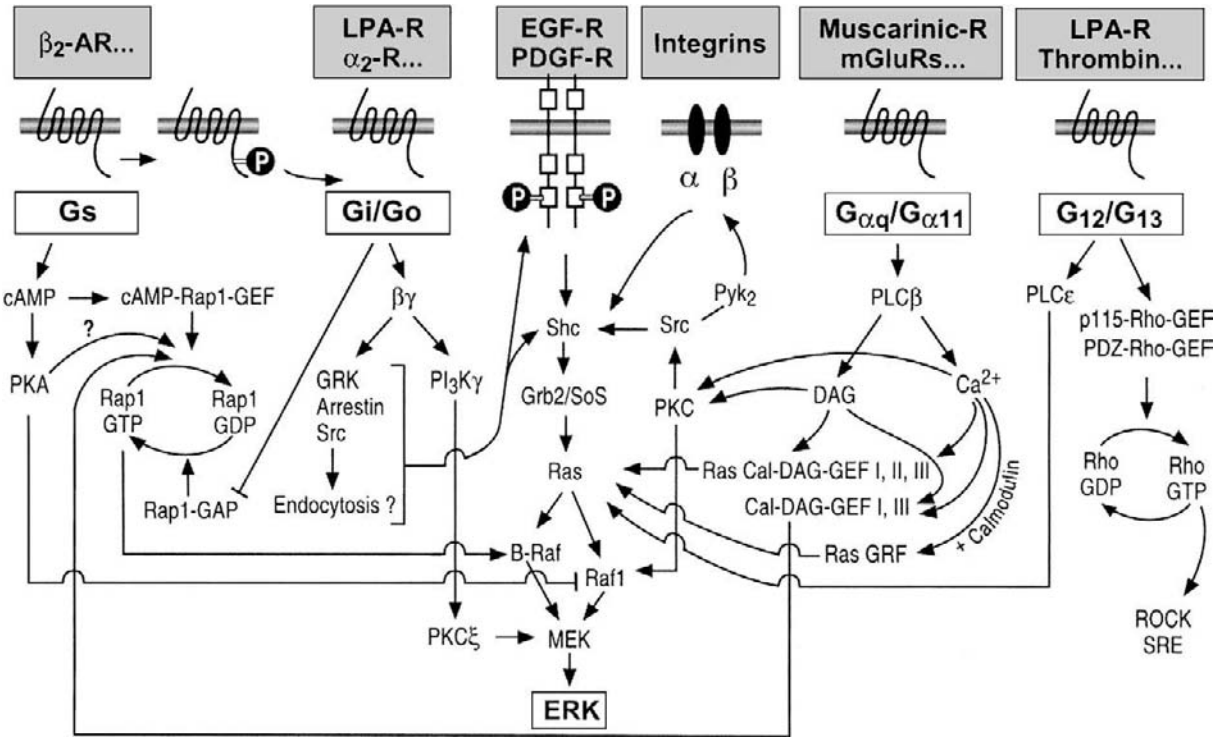


FIG. 8 GPCR receptor tyrosine kinase (RTK) dialog for activation of extracellular receptor kinases (ERKs) and cell division or differentiation. For a complete description, see Section IV.B.

TcTex-1, a dynein light-chain subunit (Tai *et al.,* 1999). This allows the transport of post-Golgi rhodopsin-containing vesicles along the microtubules up to the outer segment. β_2-AR can be endocytosed following insulin or IGF-1 phosphorylation on Tyr^{350}. This creates a SH2 binding site for Grb2 which, via its SH3 domains, recruits P13 kinase and dynamin. Induced β_2-AR internalization by insulin requires the presence of this Tyr^{350} (Karoor *et al.,* 1998). Also recall that the C terminus of dopamine D5 but not D1 receptors interacts directly with the γ_2 subunit of $GABA_A$ receptors (Liu *et al.,* 2000).

6. Proteins Interacting with the C-III Loop

As previously discussed, the C-III intracellular loop is very important for coupling to G proteins. Proteins have been shown to interact directly to C-III of GPCRs. Spinophilin interacts with C-III of D2-dopaminergic receptors (Smith *et al.,* 1999), the ξ isoform of 14-3-3 proteins with α_2-AR (Prézeau *et al.,* 1999), and endophilins with β_1-AR. Spinophilin is a protein containing an N-terminus actin binding domain, a central PDZ domain, and a CC C-terminus domain. The C-III of D2-dopaminergic receptors interacts with the domain of spinophilin localized between the actin binding domain and the PDZ domain which also binds protein phosphatase-1. It is interesting to note that two isoforms of D2 receptors are generated by alternative splicing. They differ by the presence (D2L) or absence (D2S) of 29 amino acids. It has recently been shown that D2L acts mainly at postsynaptic sites (Usiello *et al.,* 2000). Endophilins 1/2/3, comprising an SH3 domain-containing family, interact with a C-III polyproline domain of β_1-AR not present in β_2-AR. This interaction may be involved in internalization and desensitization of β_1-AR. The roles of these proteins remain to be elucidated.

7. Proteins Interacting with TM Domains

There is much evidence for regulation of small G proteins by heterotrimeric G proteins, such as Ras, Rab, Rho, and ARF, but this generally occurs via a signaling cascade localized downstream of GPCRs. However, Rho and ARF have been shown to be immunoprecipitated in an agonist-dependent manner in association with m3 muscarinic receptors and AT1 angiotensin receptors. The common receptor sequence (NPxxY) required for the small G protein interaction is localized at the end of the TM-VII and is also required to activate phospholipase D via these receptors. Direct interaction is likely but remains to be demonstrated (Mitchell *et al.,* 1998). We previously discussed the interactions of the TM domains with other GPCRs in a homomeric or heterotrimeric manner as well as with one TM protein (see Section II.C).

B. Cross Talk between the GPCRs and the Tyrosine Receptor Kinase and Small G Proteins

In association with tyrosine receptor kineses (TRKs) or alone, GPCRs and heterotrimeric G proteins have been recognized to control proliferation, differentiation, and even transformation (Gudermann *et al.,* 2000) (Fig. 8). Two main signaling pathways are involved in these functions—the Ras and Rho pathways. TRKs are classically involved in activating the Ras pathway. The cascade includes autotyrosine phosphorylation of the receptors, tyrosine phosphorylation and association with proteins such as Shc (SH2 domain-containing α_2-collagen-related) and Grb2 (growth factor-bound protein 2), and recruitment of exchange factors for Ras-like SOS. Ras-GTP engaged the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein kinases. This includes association with Raf1 or B-Raf kinases. Raf kinases are serine/threonine kinases which phosphorylate MEK (ERK kinase), which subsequently phosphorylate ERKs. Translocation of ERKs into the nucleus controls the transcription of genes involved in division or differentiation. The kinetics of ERK activation, the concomitant activation of Rap1, another small G protein, and the absence or the presence of B-Raf determine whether a TRK will induce proliferation (like epidermal growth factor and platelet-derived growth factor) or differentiation (like nerve growth factor) (Gudermann *et al.,* 2000). GPCRs acting via the four main subfamilies of heterotrimeric G proteins—Gs, Gi/Go, Gq/G11, and G12/G13—control the TRK–ERK kinase pathway.

1. Gs-Coupled Receptors

We previously discussed the fact that cAMP in some cells induces cell proliferation, whereas in many others cAMP is inhibitory. In cells such as thyroid epithelium and GH-secreting cells, gain of function of Gs leads to adenoma formation. In fact, cAMP via PKA may either inhibit Raf1 and therefore ERK and cell proliferation, like in NIH-3T3 fibroblasts (Chen and Iyengar, 1994), or activate ERK via activation of Rap1 and B-Raf (Vossler *et al.,* 1997). In neuronal cells B-Raf is the most important MEK activator and cAMP always activates ERK. cAMP seems to activate Rap1 without requiring the activation of PKA. Indeed, a cAMP Rap1 guanine nucleotide exchange factor which is directly activated by cAMP, also named Epac (exchange protein directly activated by cAMP), has been described (de Rooij *et al.,* 1998; Kawasaki *et al.,* 1998). β_2-AR activates ERK via Rap1 and B-Raf in HEK cells (Schmitt and Stork, 2000).

2. Gi/Go-Coupled Receptors

Descriptions of tumors induced by constitutively active Gαi are very limited and are described only in human ovarian sex cord stromal tumors and adrenal cortical tumors (Gudermann *et al.,* 2000). However, many GPCRs, such as lysophosphatidic

acid (LPA) receptors and α_2-AR and 5-HT_{1A} receptors, have been shown to activate the ERK pathway and cell division very efficiently via PTX-dependent Gi/Go (Gudermann *et al.,* 2000; Varrault *et al.,* 1992). In addition, the β_2-AR Gs-coupled GPCR has also been shown to activate the ERK pathway via Gi/Go after its agonist induced phosphorylation (Daaka *et al.,* 1997). G$\beta\gamma$ is the active dimer after receptor activation. Several possible pathways are conceivable. We previously discussed the fact that G$\beta\gamma$ binds GRK, leading to receptor phosphorylation, binding of arrestin, and recruitment of Src and clathrin. The endocytosis of the complex, via coated pits, conveys Src in the cytoplasm. Src phosphorylates Sch, which is a link to the Ras–ERK cascade (Luttrell *et al.,* 1998). However, some Gi/Go-coupled GPCRs, such as GnRH, activate ERK probably without requiring arrestin. Using dominant-negative dynamin mutants which block endocytosis, some but not all authors have reported inhibition of GPCR or EGF-mediated ERK activation (Tsao and Zastrow, 2000). Therefore, the requirement for endocytosis may not be a generalized mechanism of ERK activation by GPCRs. G$\beta\gamma$, which also activates P13 kinase γ, may directly activate MEK via PKC-ζ (Takeda *et al.,* 1999). GPCRs such as angiotensin AT1, LPA, and thrombin receptors can also transphosphorylate RTKs via Src and unknown kinases (Heeneman *et al.,* 2000; Zwick *et al.,* 1999). Gαo/i may also activate the Rap1 pathway by sequestering Rap–GAP (Mochizuki *et al.,* 1999). G$\beta\gamma$ also activates Cdc42 (Simon *et al.,* 1995) and binds Rho and Rac (Harhammer *et al.,* 1996) and Arf (ADP ribosylation factor) (Franco *et al.,* 1995), which is involved in coat formation and vesicular trafficking.

3. Gq/G11-Coupled Receptors

There are many pathways by which Gq/G11-activating GPCRs can activate the ERK pathway (Gudermann *et al.,* 2000). RasCalDAG-GEFI, and -II, -III and Ras guanine nucleotide exchange factor (GEF; also called GRF—GDP releasing factor) predominantly expressed in the nervous system, are activated by diacylglycerol (DAG) and Ca^{2+} (Yamashita *et al.,* 2000), whereas Ras GRF is a Ras GEF activated by Ca^{2+} calmodulin in neuronal cells (Mattingly and Macara, 1996). In addition, CalDAG–GEFI and -III are also Rap1 GEF (Yamashita *et al.,* 2000). PKC has been shown to directly activate Raf-1 and the PYK2–Src interaction (Dikic *et al.,* 1996).

4. G12/G13-Coupled Receptors

Human G12 has been identified as an oncogenic protein in NIH-3T3 cells (Chan *et al.,* 1993). GTPase-deficient Gα12 and Gα13 have been reported by several laboratories to be very efficient in transforming proteins. Gα12 has been identified as a transforming oncogene in Ewing's sarcoma, and the term gep oncogene has been attributed to Gα12 (Xu *et al.,* 1994). GPCR-mediated activation of G13 (and to a lesser extent G12) leads to p115RHoGEF, a regulator of G protein signaling for these heterotrimeric G proteins and a Rho GEF factor (Hart *et al.,* 1998; Kozasa

et al., 1998). Similarly, another G12/G13 RGS (PDZ-RhoGEF) is also a Rho GEF (Fukuhara *et al.,* 1999). GPCR-induced Rho activation may induce cytoskeletal rearrangements. However, the mechanisms by which this activation of Rho control growth proliferation remain unclear. In some cases, such as LPA, bradykinin (B2), and serotonin (5-HT_2) GPCRs, the involvement of EGF receptors is required to activate Rho. Rho regulates transcriptional events via serum-responsive element and activates ROCK-I (Rho-associated CC-forming kinase), two events which are likely to play a role in cell division (Gudermann *et al.,* 2000). In addition, G12 interacts directly with PLC-ε, a novel PLC member which is a Ras GEF (Lopez *et al.,* 2001).

V. Concluding Remarks

The GPCR saga started 25 years ago with a very simple question: How do hormones act at the cellular level? Thus, the chapter on cellular signaling was opened. Landmarks of this saga were the discovery of cAMP, the discovery and purification of heterotrimeric G proteins, and the purification and cloning of GPCRs, and, finally, the crystal structure of rhodopsin. Cellular signaling and cell–cell communication are now the central question in biology which will remain until physiological functions and regulations, particularly brain functions (the cell–cell communication problem) and pathological diseases such as cancer, are fully understood.

Acknowledgments

We thank F. Carroll, T. Galvez, V. Homburger, L. Joubert, M. Passama, and Angie Turner-Madeuf for their help in preparing the manuscript and for fruitful discussions.

References

AbdAlla, S., Zaki, E., Lother, H., *et al.* (1999). Involvement of the amino terminus of the B(2) receptor in agonist-induced receptor dimerization. *J. Biol. Chem.* **274,** 26079–26084.

AbdAlla, S., Lother, H., and Quitterer, U. (2000). AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature* **407,** 94–98.

Adler, E., Hoon, M. A., Mueller, K. L., *et al.* (2000). A novel family of mammalian taste receptors. *Cell* **100,** 693–702.

Ahn, S., Maudsley, S., Luttrell, L. M., *et al.* (1999). Src-mediated tyrosine phosphorylation of dynamin is required for beta 2-adrenergic receptor internalization and mitogen-activated protein kinase signaling. *J. Biol. Chem.* **274,** 1185–1188.

Ali, M. S., Sayeski, P. P., Dirksen, L. B., *et al.* (1997). Dependence on the motif YIPP for the physical association of Jak2 kinase with the intracellular carboxyl tail of the angiotensin II AT1 receptor. *J. Biol. Chem.* **272,** 23382–23388.

Ames, R. S., Sarau, H. M., Chambers, J. K., *et al.* (1999). Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14 [published erratum appears in *Nature* 1999 Dec 23–30; **402**(6764):898]. *Nature* **401,** 282–286.

Angers, S., Salahpour, A., Joly, E., *et al.* (2000). Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. USA* **97,** 3684–3689.

Ango, F., Pin, J. P., Tu, J. C., *et al.* (2000). Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by Homer 1 proteins and neuronal excitation. *J. Neurosci.* **20,** 8710–8716.

Ango, F., Prézeau, L., Muller, T., *et al.* (2001). Agonist-independent activation of mGluRs by the intracellular protein, Homer. *Nature,* **411,** 962–965.

Antonny, B., Otto-Bruc, A., Chabre, M., *et al.* (1993). GTP hydrolysis by purified alpha-subunit of transducin and its complex with the cGMP phosphodiesterase inhibitor. Biochemistry, **32,** 8646–8653.

Armstrong, N., Sun, Y., Chen, G. Q., *et al.* (1998). Structure of a glutamate–receptor ligand-binding core in complex with kainate. *Nature* **395,** 913–917.

Arnis, S., Fahmy, K., Hofmann, K. P., *et al.* (1994). A conserved carboxylic acid group mediates light-dependent proton uptake and signaling by rhodopsin. *J. Biol. Chem.* **269,** 23879–23881.

Arshavsky, V., and Bownds, M. D. (1992). Regulation of deactivation of photoreceptor G protein by its target enzyme and cGMP. *Nature* **357,** 416–417.

Arthus, M. F., Lonergan, M., Crumley, M. J., *et al.* (2000). Report of 33 novel AVPR2 mutations and analysis of 117 families with X-linked nephrogenic diabetes insipidus. *J. Am. Soc. Nephrol.* **11,** 1044–1054.

Backstrom, J. R., Price, R. D., Reasoner, D. T., *et al.* (2000). Deletion of the serotonin 5-HT2C receptor PDZ recognition motif prevents receptor phosphorylation and delays resensitization of receptor responses. *J. Biol. Chem.* **275,** 23620–23626.

Bahner, M., Sander, P., Paulsen, R., *et al.* (2000). The visual G protein of fly photoreceptors interacts with the PDZ domain assembled INAD signaling complex via direct binding of activated Galpha(q) to phospholipase cbeta. *J. Biol. Chem.* **275,** 2901–2904.

Bai, M., Trivedi, S., and Brown, E. M. (1998). Dimerization of the extracellular calcium-sensing receptor (CaR) on cell surface of CaR-transfected HEK293 cells. *J. Biol. Chem.* **273,** 23605–23610.

Bai, M., Trivedi, S., Kifor, O., *et al.* (1999). Intermolecular interactions between dimeric calcium-sensing receptor monomers are important for its normal function. *Proc. Natl. Acad. Sci. USA* **96,** 2834–2839.

Baker, E., Colley, N., and Zuker, C. (1994). The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *EMBO J.* **13,** 4886–4895.

Baldwin, J. M. (1993). The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12,** 1693–1703.

Ballesteros, J., Kitanovic, S., Guarnieri, F., *et al.* (1998). Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **273,** 10445–10453.

Baranski, T. J., Herzmark, P., Lichtarge, O., *et al.* (1999). C5a receptor activation. Genetic identification of critical residues in four transmembrane helices. *J. Biol. Chem.* **274,** 15757–15765.

Barber, D. L., Ganz, M. B., Bongiorno, P. B., *et al.* (1992). Mutant constructs of the beta-adrenergic receptor that are uncoupled from adenylyl cyclase retain functional activation of Na–H exchange. *Mol. Pharmacol.* **41,** 1056–1060.

Bargmann, C. (1997). Olfactory receptors, vomeronasal receptors, and the organization of olfactory information. *Cell* **90,** 585–587.

Bargmann, C. (1998). Neurobiology of the *Caenorhabditis elegans* genome. *Science* **282,** 2028–2033.

Barritt, D. S., Pearn, M. T., Zisch, A. H., *et al.* (2000). The multi-PDZ domain protein MUPP1 is a cytoplasmic ligand for the membrane-spanning proteoglycan NG2. *J. Cell Biochem.* **79,** 213–224.

Bécamel, C., Figge, A., Poliak, S., *et al.* (2001). Interaction of serotonin 5-HT2C receptors with PDZ10 of the multi PDZ protein MUPP1. *J. Biol. Chem.,* **276,** 12974–12982.

Beneken, J., Tu, J. C., Xiao, B., *et al.* (2000). Structure of the Homer EVH1 domain–peptide complex reveals a new twist in polyproline recognition. *Neuron* **26,** 143–154.

Benkirane, M., Jin, D. Y., Chun, R. F., *et al.* (1997). Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J. Biol. Chem.* **272,** 30603–30606.

Benzing, T., Brandes, R., Sellin, L., *et al.* (1999). Upregulation of RGS7 may contribute to tumor necrosis factor-induced changes in central nervous function. *Nat. Med.* **5,** 913–918.

Berg, K. A., Stout, B. D., Cropper, J. D., *et al.* (1999). Novel actions of inverse agonists on 5-HT2C receptor systems. *Mol. Pharmacol.* **55,** 863–872.

Berger, E. A., Murphy, P. M., and Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: Roles in viral entry tropism, and disease. *Annu. Rev. Immunol.* **17,** 657–700.

Berke, J. D., Paletzki, R. F., Aronson, G. J., *et al.* (1998). A complex program of striatal gene expression induced by dopaminergic stimulation. *J. Neurosci.* **18,** 5301–5310.

Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996). GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. *Cell* **86,** 445–452.

Berstein, G., Blank, J., John, D.-Y., *et al.* (1992). Phospholipase C-β1 is a GTPase-activating protein for Gq/11, its physiologic regulator. *Cell* **70,** 411–418.

Bessis, A.-S., Bertrand, H. O., Galvez, T., *et al.* (2000). Three dimensional model of the extracellular domain of the type 4a metabotropic glutamate receptor: New insights into the activation process. *Protein Sci.* **9,** 2200–2209.

Betancur, C., Azzi, M., and Rostène, W. (1997). Nonpeptide antagonists of neuropeptide receptors: Tools for research and therapy. *Trends Pharmacol. Sci* **18,** 372–385.

Bidot, S., Bidot, C., Spassky, N., *et al.* (2000). RGS14 is a novel Rap effector that preferentially regulates the GTPase activity of Galpha o. *Biochem. J.* **350,** 19–29.

Birnbaumer, L., and Rodbell, M. (1969). Adenylyl cyclase in fat cells. II. Hormone receptors. *J. Biol. Chem.* **344,** 3477–3482.

Birnbaumer, M. (1995). Mutations and diseases of G protein coupled receptors. *J. Receptor Signal Transduction Res.* **15,** 131–160.

Blahos, J., II, Mary, S., Perroy, J., *et al.* (1998). Extreme C terminus of G protein α-subunits contains a site that discriminates between G_i-coupled metabotropic glutamate receptors. *J. Biol. Chem.,* **273,** 25765–25769.

Blahos, J., II, Fischer, T., Brabet, I., *et al.* (2001). Different sites on G-protein α-subunits cooperate to control coupling to heptahelical receptors. *J. Biol. Chem.,* **276,** 3262–3269.

Bockaert, J., and Pin, J.-P. (1998). Utiliser un récepteur couplé aux protéines G pour communiquer: un succès évolutif. *C. R. Acad. Sci. Paris* **321,** 529–551.

Bockaert, J., and Pin, J. P. (1999). Molecular tinkering of G protein-coupled receptors: An evolutionary success. *EMBO J.* **18,** 1723–1729.

Boguski, M. S., and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* **366,** 643–654.

Bohm, S. K., Grady, E. F., and Bunnett, N. W. (1997). Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem. J.* **322,** 1–18.

Bohn, L., Gainetdinov, R., Lin, F.-T., *et al.* (2000). μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408,** 720–723.

Bond, R. A., Leff, P., Johnson, T. D., *et al.* (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of β2-adrenoceptor. *Nature* **374,** 272–276.

Borhan, B., Souto, M. L., Imai, H., *et al.* (2000). Movement of retinal along the visual transduction path. *Science* **288,** 2209–2212.

Bouaboula, M., Desnoyer, N., Carayon, P., *et al.* (1999). Gi protein modulation induced by a selective inverse agonist for the peripheral cannabinoid receptor CB2: Implication for intracellular signalization cross-regulation. *Mol. Pharmacol.* **55,** 473–480.

Bourne, H. (1997a). The arginine finger strikes again. *Nature* **389,** 673–674.

Bourne, H. (1997b). How receptors talk to trimeric G proteins. *Curr. Opin. Cell. Biol.* **9,** 134–142.

Bourne, H. R., and Meng, E. C. (2000). Structure, Rhodopsin sees the light. *Science* **289,** 733–734.

Brabet, P., Pantaloni, C., Bockaert, J., *et al.* (1991). Metabolism of two Goα isoforms in neuronal cells during differentiation. *J. Biol. Chem.* **266,** 12825–12828.

Braestrup, C., Schmiechen, R., Neef, G., *et al.* (1982). Interaction of convulsive ligands with benzodiazepine receptors. *Science* **216,** 1241–1243.

Brakeman, P. R., Lanahan, A. A., O'Brien, R., *et al.* (1997). Homer: A protein that selectively binds metabotropic glutamate receptors. *Nature* **386,** 284–288.

Brauner-Osborne, H., and Krogsgaard-Larsen, P. (2000). Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain. *Genomics* **65,** 121–128.

Brown, E. M. (1999). Physiology and pathophysiology of the extracellular calcium-sensing receptor. *Am. J. Med.* **106,** 238–253.

Brown, E. M., and Hebert, S. C. (1997). Calcium-receptor-regulated parathyroid and renal function. *Bone* **20,** 303–309.

Bunemann, M., and Hosey, M. M. (1999). G-protein coupled receptor kinases as modulators of G-protein signalling. *J. Physiol. (London)* **517,** 5–23.

Bunemann, M., Lee, K. B., Pals-Rylaarsdam, R., *et al.* (1999). Desensitization of G-protein-coupled receptors in the cardiovascular system. *Annu. Rev. Physiol.* **61,** 169–192.

Burns, C. M., Chu, H., Rueter, S., *et al.* (1997). Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature* **387,** 303–308.

Buscher, R., Herrmann, V., and Insel, P. A. (1999). Human adrenoceptor polymorphisms: Evolving recognition of clinical importance. *Trends Pharmacol. Sci.* **20,** 94–99.

Cao, T. T., Deacon, H. W., Reczek, D., *et al.* (1999). A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β2-adrenergic receptor. *Nature* **401,** 286–290.

Caron, P., Chauvin, S., Christin-Maitre, S., *et al.* (1999). Resistance of hypogonadic patients with mutated GnRH receptor genes to pulsatile GnRH administration. *J. Clin. Endocrinol. Metab.* **84,** 990–996.

Carroll, F., Stolle, A., Beart, P., *et al.* (2001). BAY 36-7620 is a new potent non-competitive mGlu1 receptor antagonist and the first inverse agonist on mGlu1a. *Mol. Pharmacol.* **5,** 965–973.

Chabre, M., and Deterre, P. (1989). Molecular mechanism of visual transduction. *Eur. J. Biochem.* **179,** 255–256.

Chambers, J., Ames, R. S., Bergsma, D., *et al.* (1999). Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature* **400,** 261–265.

Chan, A. M., Fleming, T. P., McGovern, E. S., *et al.* (1993). Expression cDNA cloning of a transforming gene encoding the wild-type G alpha 12 gene product. *Mol. Cell. Biol.* **13,** 762–768.

Chandrashekar, J., Mueller, K. L., Hoon, M. A., *et al.* (2000). T2Rs function as bitter taste receptors. *Cell* **100,** 703–711.

Changeux, J., and Edelstein, S. (1998). Allosteric receptors after 30 years. *Neuron* **21,** 959–980.

Chaudhari, N., Landin, A. M., and Roper, S. D. (2000). A metabotropic glutamate receptor variant functions as a taste receptor. *Nature Neurosci.* **3,** 113–119.

Chemelli, R. M., Willie, J. T., Sinton, C. M., *et al.* (1999). Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell* **98,** 437–451.

Chen, C. K., Burns, M. E., He, W., *et al.* (2000). Slowed recovery of rod photoresponse in mice lacking the GTPase accelerating protein RGS9-1. *Nature* **403,** 557–560.

Chen, J., and Iyengar, R. (1994). Suppression of Ras-induced transformation of NIH 3T3 cells by activated G alphas. *Science* **263,** 1278–1281.

Chidiac, P., Hebert, T. E., Valiquette, M., *et al.* (1994). Inverse agonist activity of β-adrenergic antagonists. *Mol. Pharmacol.* **45,** 490–499.

Cismowski, M. J., Ma, C., Ribas, C., *et al.* (2000). Activation of heterotrimeric G-protein signaling by a Ras-related protein. *J. Biol. Chem.* **275,** 23421–23424.

Claeysen, S., Sebben, M., Bécamel, C., *et al.* (1999). Novel brain-specific 5-HT4 receptor splice variants show marked constitutive activity: Role of the C-terminal intracellular domain. *Mol. Pharmacol.* **55,** 910–920.

Claeysen, S., Sebben, M., Bécamel, C., *et al.* (2000). Pharmacological properties of the 5-hydroxytryptamine 4 receptor antagonists on constitutively active wild-type and mutated receptors. *Mol. Pharmacol.* **58,** 136–144.

Claeysen, S., Sebben, M., Bécamel, C., *et al.* (2001). Constitutively active mutants of 5-HT4 receptors: Are they in unique active states?. *EMBO Rep.* **2,** 61–67.

Clapham, D. E., and Neer, E. J. (1993). New roles for G-protein $\beta\gamma$-dimers in transmembrane signaling. *Nature* **365,** 403–406.

Clark, A. J. (1933). *The Mode of Action of Drugs on Cells.* Arnold, London.

Clark, A. J., McLoughlin, L., and Grossman, A. (1993). Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. *Lancet* **341,** 461–462.

Clyne, P. J., Warr, C. G., Freeman, M. R., *et al.* (1999). A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in *Drosophila*. *Neuron* **22,** 327–338.

Clyne, P. J., Warr, C. G., and Carlson, J. R. (2000). Candidate taste receptors in *Drosophila*. *Science* **287,** 1830–1834.

Cohen, G. B., Yang, T., Robinson, P. R., *et al.* (1993). Constitutive activation of opsin: Influence of charge at position 134 and size at position 296. *Biochemistry* **32,** 6111–6115.

Coleman, D. E., Berghuis, A. M., Lee, E., *et al.* (1994). Structures of active conformations of Giα_1 and the mechanism of GTP hydrolysis. *Science* **265,** 1405–1412.

Conklin, B. R., Farfel, Z., Lustig, K. D., *et al.* (1993). Substitution of three amino acids switches receptor specificity of Gqα to that of Giα. *Nature* **363,** 274–276.

Costa, T., and Hertz, A. (1989). Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* **86,** 7321–7325.

Cotecchia, S., Exum, S., Caron, M. G., *et al.* (1990). Regions of the α1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc. Natl. Acad. Sci. USA* **87,** 2896–2900.

Cotecchia, S., Ostrowski, J., Kjelsberg, J., *et al.* (1992). Discrete amino acid sequences of the alpha 1-adrenergic receptor determine the selectivity of coupling to phosphatidylinositol hydrolysis. *J. Biol. Chem.* **267,** 1633–1639.

Coughlin, S. R. (1999). How the protease thrombin talks to cells. *Proc. Natl. Acad. Sci. USA* **96,** 11023–11027.

Cvejic, S., and Devi, L. A. (1997). Dimerization of the delta opioid receptor: Implication for a role in receptor internalization. *J. Biol. Chem.* **272,** 26959–26964.

Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997). Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390,** 88–91.

Daniels, D. L., Cohen, A. R., Anderson, J. M., *et al.* (1998). Crystal structure of the hCASK PDZ domain reveals the structural basis of class II PDZ domain target recognition. *Nat. Struct. Biol.* **5,** 317–325.

Daviet, L., Lehtonen, J. Y., Tamura, K., *et al.* (1999). Cloning and characterization of ATRAP, a novel protein that interacts with the angiotensin II type 1 receptor. *J. Biol. Chem.* **274,** 17058–17062.

Davletov, B., Meunier, F., Ashton, A., *et al.* (1998). Vesicle exocytosis stimulated by alpha-latrotoxin is mediated by latrophilin and requires both external and stored Ca^{2+}. *EMBO J.* **17,** 3909–3920.

De Clercq, E. (2000). Inhibition of HIV infection by bicyclams, highly potent and specific CXCR4 antagonists. *Mol. Pharmacol.* **57,** 833–839.

Defer, N., Best-Belpomme, M., and Hanoune, J. (2000). Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *Am. J. Physiol. Renal Physiol.* **279,** F400–F416.

De Lean, A., Stadel, J. M., and Lefkowitz, R. J. (1980). A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase. *J. Biol. Chem.* **255,** 7108–7117.

de Ligt, R. A. F., Kourounakis, A. P., and Ijzerman, A. P. (2000). Inverse agonism at G protein-coupled receptors: (Patho)physiological relevance and implications in drug discovery. *Br. J. Pharmacol.* **130,** 1–12.

de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., *et al.* (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396,** 474–477.

de Roux, N., Young, J., Brailly-Tabard, S., *et al.* (1999a). The same molecular defects of the gonadotropin-releasing hormone receptor determine a variable degree of hypogonadism in affected kindred. *J. Clin. Endocrinol. Metab.* **84,** 567–572.

de Roux, N., Young, J., Misrahi, M., *et al.* (1999b). Loss of function mutations of the GnRH receptor: A new cause of hypogonadotropic hypogonadism. *J. Pediatr. Endocrinol. Metab.* **12**(Suppl 1), 267–275.

Dev, K. K., Nakajima, Y., Kitano, J., *et al.* (2000). PICK1 interacts with and regulates PKC phosphorylation of mGluR7. *J. Neurosci.* **20,** 7252–7257.

Devreotes, P. (1994). G protein-linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron* **12,** 235–241.

De Vries, L., Mousli, M., Wurmser, A., *et al.* (1995). GAIP, a protein that specifically interacts with the trimeric G protein Gαi3, is a member of a protein family with a highly conserved core domain. *Proc. Natl. Acad. Sci. USA* **1995,** 11916–11920.

De Vries, L., Zheng, B., Fischer, T., *et al.* (2000). The regulation of G protein signaling family. *Annu. Rev. Pharmacol. Toxicol.* **40,** 235–271.

Dikic, I., Tokiwa, G., Lev, S., *et al.* (1996). A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383,** 547–550.

Dohlman, H. G., and Thorner, J. (1997). RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.* **272,** 3871–3874.

Dohlman, H. G., Thorner, J., Caron, M. G., *et al.* (1991). Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60,** 653–688.

Doupnik, C., Davidson, N., Lester, H. A., *et al.* (1997). RGS proteins reconstitute the rapid gating kinetics of G$\beta\gamma$-activated inwardly rectifying K^+ channels. *Proc. Natl. Acad. Sci. USA* **94,** 10461–10466.

Druey, K. M., and Kehrl, J. H. (1997). Inhibition of regulator of G protein signaling function by two mutant RGS4 proteins. *Proc. Natl. Acad. Sci. USA* **94,** 12851–12856.

Dryja, T. P., and Li, T. (1995). Molecular genetics of retinitis pigmentosa. *Hum. Mol. Genet.* **4,** 1739–1743.

Duprez, L., Parma, J., Van Sande, J., *et al.* (1999). Pathology of the TSH receptor. *J. Pediatr. Endocrinol. Metab.* **12**(Suppl 1), 295–302.

Dwyer, N., Troemel, E., Sengupta, P., *et al.* (1998). Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. *Cell* **93,** 455–466.

Ebstein, R. P., Novick, O., Umansky, R., *et al.* (1996). Dopamine D4 receptor (D4DR) exon III polymorphism associated with the human personality trait of Novelty Seeking. *Nat. Genet.* **12,** 78–80.

Edwards, S. W., Tan, C. M., and Limbird, L. E. (2000). Localization of G-protein-coupled receptors in health and disease. *Trends Pharmacol. Sci.* **21,** 304–308.

Ehlers, M. D., Fung, E. T., O'Brien, R. J., *et al.* (1998). Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments. *J. Neurosci.* **18,** 720–730.

Elling, C. E., Moller Nielsen, S., and Schwartz, T. W. (1995). Conversion of antagonist-binding site to metal-ion site in tachykinin NK-1 receptor. *Nature* **374,** 74–77.

Fagni, L., Chavis, P., Ango, F., *et al.* (2000). Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. *Trends Neurosci.* **23,** 80–88.

Farrens, D. L., Altenbach, C., Yang, K., *et al.* (1996). Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science* **274,** 768–770.

Faure, S., Meyer, L., Costagliola, D., *et al.* (2000). Rapid progression to AIDS in HIV^+ individuals with a structural variant of the chemokine receptor CX3CR1. *Science* **287,** 2274–2277.

Ferreira, P., Nakayama, T., Pak, W., *et al.* (1996). Cyclophilin-related protein RanBP2 acts as chaperone for red/green opsin. *Nature* **383,** 637–640.

Franco, M., Paris, S., and Chabre, M. (1995). The small G-protein ARF1GDP binds to the Gt beta gamma subunit of transducin, but not to Gt alpha GDP-Gt beta gamma. *FEBS Lett.* **362,** 286–290.

Fraser, C. M., and Venter, J. C. (1982). The size of the mammalian lung beta 2-adrenergic receptor as determined by target size analysis and immunoaffinity chromatography. *Biochem. Biophys. Res. Commun.* **109,** 21–29.

Fukuhara, S., Murga, C., Zohar, M., *et al.* (1999). A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. *J. Biol. Chem.* **274,** 5868–5879.

Galvez, T., Prézeau, L., Milioti, G., *et al.* (2000a). Mapping the agonist binding site of GABA B type 1 receptor: Insights into the activation process of family 3 heptahelix receptors. *J. Biol. Chem.* **275,** 41166–41174.

Galvez, T., Urwyler, S., Prézeau, L., *et al.* (2000b). Ca(2+) requirement for high-affinity gamma-aminobutyric acid (GABA) binding at GABA(B) receptors: Involvement of serine 269 of the GABA(B)R1 subunit. *Mol. Pharmacol.* **57,** 419–426.

Galvez, T., Duthey, B., Kniazeff, J., *et al.* (2001). Allosteric interactions between GB_1 and GB_2 subunits are required for optimal GABA (B) receptor activation. *EMBO J.* **20,** 2152–2159.

Gao, Q., and Chess, A. (1999). Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. *Genomics* **60,** 31–39.

George, S. R., Lee, S. P., Varghese, G., *et al.* (1998). A transmembrane domain-derived peptide inhibits D1 dopamine receptor function without affecting receptor oligomerization. *J. Biol. Chem.* **273,** 30244–30248.

George, S. R., Fan, T., Xie, Z., *et al.* (2000). Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J. Biol. Chem.* **275,** 26128–26135.

Geras-Raaka, E., Varma, A., Ho, H., *et al.* (1998). Human interferon-gamma-inducible protein 10 (IP-10) inhibits constitutive signaling of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *J. Exp. Med.* **188,** 405–408.

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrinol. Rev.* **21,** 90–113.

Gether, U., Johansen, T. E., Snider, R. M., *et al.* (1993). Different binding epitopes on the NK1 receptor for substance P and a non-peptide antagonist. *Nature* **362,** 345–348.

Gether, U., Ballesteros, J. A., Seifert, R., *et al.* (1997a). Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility. *J. Biol. Chem.* **272,** 2587–2590.

Gether, U., Lin, S., Ghanouni, P., *et al.* (1997b). Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. *EMBO J.* **16,** 6737–6747.

Gilman, A. G. (1987). G proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56,** 615–649.

Godowski, P. J., Leung, D. W., Meacham, L. R., *et al.* (1989). Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron-type dwarfism. *Proc. Natl. Acad. Sci. USA* **86,** 8083–8087.

Goldsmith, P. K., Fan, G. F., Ray, K., *et al.* (1999). Expression, purification, and biochemical characterization of the amino-terminal extracellular domain of the human calcium receptor. *J. Biol. Chem.* **274,** 11303–11309.

Golser, R., Gorren, A. C., Leber, A., *et al.* (2000). Interaction of endothelial and neuronal nitric-oxide synthases with the bradykinin B2 receptor. Binding of an inhibitory peptide to the oxygenase domain blocks uncoupled NADPH oxidation [published erratum appears in *J. Biol. Chem.* 2000 May 26; **275**(21):16400]. *J. Biol. Chem.* **275,** 5291–5296.

Gomeza, J., Joly, C., Kühn, R., *et al.* (1996a). The second intracellular loop of mGluR1 cooperates with the other intracellular domains to control coupling to G-protein. *J. Biol. Chem.* **271,** 2199–2205.

Gomeza, J., Mary, S., Brabet, I., *et al.* (1996b). Coupling of metabotropic glutamate receptors 2 and 4 to $G\alpha 15$, $G\alpha 16$ and chimeric $G\alpha q/i$ proteins: Characterization of new antagonists. *Mol. Pharmacol.* **50,** 923–930.

Grazzini, E., Guillon, G., Mouillac, B., *et al.* (1998). Inhibition of oxytocin receptor function by direct binding of progesterone. *Nature* **392,** 509–512.

Groblewski, T., Maigret, B., Larguier, R., *et al.* (1997). Mutation of Asn111 in the third transmembrane domain of the ATlA angiotensin II receptor induces its constitutive activation. *J. Biol. Chem.* **272,** 1822–1826.

Gudermann, T., Grosse, R., and Schultz, G. (2000). Contribution of receptor/G protein signaling to cell growth and transformation. *Naunyn Schmiedebergs Arch. Pharmacol.* **361,** 345–362.

Guillet-Deniau, I., Burnol, A. F., and Girard, J. (1997). Identification and localization of a skeletal muscle secrotonin 5-HT2A receptor coupled to the Jak/STAT pathway. *J. Biol. Chem.* **272,** 14825–14829.

Hager, J., Hansen, L., Vaisse, C., *et al.* (1995). A missense mutation in the glucagon receptor gene is associated with non-insulin-dependent diabetes mellitus. *Nat. Genet.* **9,** 299–304.

Hall, R., Premont, R., Chow, C., *et al.* (1998a). The beta$_2$-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. *Nature* **392,** 626–630.

Hall, R. A., Ostedgaard, L. S., Premont, R. T., *et al.* (1998b). A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na^+/H^+ exchanger regulatory factor family of PDZ proteins. *Proc. Natl. Acad. Sci. USA* **95,** 8496–8501.

Hall, R. A., Premont, R. T., and Lefkowitz, R. J. (1999). Heptahelical receptor signaling: Beyond the G protein paradigm. *J. Cell Biol.* **145,** 927–932.

Hamm, H. (1998). The many faces of G protein signaling. *J. Biol. Chem.* **273,** 669–672.

Hammerland, L. G., Krapcho, K. J., Garrett, J. E., *et al.* (1999). Domains determining ligand specificity for Ca^{2+} receptors. *Mol. Pharmacol.* **55,** 642–648.

Harhammer, R., Gohla, A., and Schultz, G. (1996). Interaction of G protein Gbetagamma dimers with small GTP-binding proteins of the Rho family. *FEBS Lett.* **399,** 211–214.

Hart, A. C., Kramer, H., Van Vactor, D. L., Jr., *et al.* (1990). Induction of cell fate in the *Drosophila* retina: The bride of sevenless protein is predicted to contain a large extracellular domain and seven transmembrane segments. *Genes Dev.* **4,** 1835–1847.

Hart, M. J., Jiang, X., Kozasa, T., *et al.* (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. *Science* **280,** 2112–2114.

Hasegawa, H., Negishi, M., and Ichikawa, A. (1996). Two isoforms of the prostaglandin E receptor EP3 subtype different in agonist-independent constitutive activity. *J. Biol. Chem.* **271,** 1857–1860.

He, W., Cowan, C. W., and Wensel, T. G. (1998). RGS9, a GTPase accelerator for phototransduction. *Neuron* **20,** 95–102.

Hebert, T. E., Moffett, S., Morello, J. P., *et al.* (1996). A peptide derived from a β_2AR transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem.* **271,** 16384–16392.

Heeneman, S., Haendeler, J., Saito, Y., *et al.* (2000). Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor beta receptor. Key role for the p66 adaptor protein Shc. *J. Biol. Chem.* **275,** 15926–15932.

Herbert, T. E., Moffett, S., Morello, J. P., *et al.* (1996). A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem.* **271,** 16384–16392.

Hinney, A., Schmidt, A., Nottebom, K., *et al.* (1999). Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. *J. Clin. Endocrinol. Metab.* **84,** 1483–1486.

Hinuma, S., Habata, Y., Fujii, R., *et al.* (1998). A prolactin-releasing peptide in the brain [published erratum appears in *Nature* 1998 Jul 16;**394**(6690):302]. *Nature* **393,** 272–276.

Hinuma, S., Onda, H., and Fujino, M. (1999). The quest for novel bioactive peptides utilizing orphan seven-transmembrane-domain receptors. *J. Mol. Med.* **77,** 495–504.

Hirata, T., Kakizuka, A., Ushikubi, F., *et al.* (1994). Arg60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. *J. Clin. Invest.* **94,** 1662–1667.

Hoon, M. A., Adler, E., Lindemeier, J., *et al.* (1999). Putative mammalian taste receptors: A class of taste-specific GPCRs with distinct topographic selectivity. *Cell* **96,** 541–551.

Houssami, S., Findlay, D. M., Brady, C. L., *et al.* (1994). Isoforms of the rat calcitonin receptor: Consequences for ligand binding and signal transduction. *Endocrinology* **135,** 183–190.

Hsu, S. Y., Liang, S. G., and Hsueh, A. J. (1998). Characterization of two LGR genes homologous to gonadotropin and thyrotropin receptors with extracellular leucine-rich repeats and a G protein-coupled, seven-transmembrane region. *Mol. Endocrinol.* **12,** 1830–1845.

Innamorati, G., Sadeghi, H., and Birnbaumer, M. (1999). Phosphorylation and recycling kinetics of G protein-coupled receptors. *J. Receptor Signal Transduction Res.* **19,** 315–326.

Javitch, J. A., Fu, D., Liapakis, G., *et al.* (1997). Constitutive activation of the β_2-adrenergic receptor alters the orientation of its sixth membrane-spanning segment. *J. Biol. Chem.* **272,** 18546–18549.

Joly, C., Gomeza, J., Brabet, I., *et al.* (1995). Molecular, functional and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: Comparison with mGluR1. *J. Neurosci.* **15,** 3970–3981.

Jones, K., Borowsky, B., Tamm, J., *et al.* (1998a). Cloning of $GABA_B$R2: Coexpression with $GABA_B$R1 permits GIRK activation in oocytes and mammalian cells. *Soc. Neurosci. Abstr.* **24,** 1991.

Jones, K. A., Borowsky, B., Tamm, J. A., *et al.* (1998b). $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_B$R1 and $GABA_B$R2. *Nature* **396,** 674–679.

Jordan, B. A., and Devi, L. A. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* **399,** 697–700.

Josefsson, L. G. (1999). Evidence for kinship between diverse G-protein coupled receptors. *Gene* **239,** 333–340.

Josefsson, L. G., and Rask, L. (1997). Cloning of a putative G-protein-coupled receptor from *Arabidopsis thaliana. Eur. J. Biochem.* **249,** 415–420.

Ju, H., Venema, V. J., Marrero, M. B., *et al.* (1998). Inhibitory interactions of the bradykinin B2 receptor with endothelial nitric-oxide synthase. *J. Biol. Chem.* **273,** 24025–24029.

Jung, H., Windhaber, R., Palm, D., *et al.* (1996). Conformation of a β-adrenoceptor-derived signal transducing peptide as inferred by circular dichroism and 1H NMR spectroscopy. *Biochemistry* **35,** 6399–6405.

Kajava, A. V., Vassart, G., and Wodak, S. J. (1995). Modeling of the three-dimensional structure of proteins with the typical leucine-rich repeats. *Structure* **3,** 867–877.

Kammerer, R. A., Frank, S., Schulthess, T., *et al.* (1999). Heterodimerization of a functional GABA B receptor is mediated by parallel coiled-coil alpha-helices. *Biochemistry* **38,** 13263–13269.

Karoor, V., Wang, L., Wang, H. Y., *et al.* (1998). Insulin stimulates sequestration of beta-adrenergic receptors and enhanced association of beta-adrenergic receptors with Grb2 via tyrosine 350. *J. Biol. Chem.* **273,** 33035–33041.

Kato, A., Ozawa, F., Saitoh, Y., *et al.* (1997). vesl, a gene encoding VASP/Ena family related protein is upregulated during seizure, long-term potentiation and synaptogenesis. *FEBS Lett.* **412,** 183–189.

Kato, A., Ozawa, F., Saitoh, Y., *et al.* (1998). Novel members of the Vesl/Homer family of the PDZ proteins that bind metabotropic glutamate receptors. *J. Biol. Chem.* **273,** 23969–23975.

Kaupmann, K., Huggel, H., Heid, J., *et al.* (1997). Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. *Nature* **386,** 239–246.

Kaupmann, K., Malitschek, B., Schuler, V., *et al.* (1998). $GABA_B$ receptor subtypes assemble into functional heteromeric complexes. *Nature* **396,** 683–687.

Kawabata, S., Tsutsumi, R., Kohara, A., *et al.* (1996). Control of calcium oscillation of metabotropic glutamate receptors. *Nature* **383,** 89–92.

Kawasaki, H., Springett, G., Toki, S., *et al.* (1998). A rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proc. Natl. Acad. Sci. USA* **95,** 1378–13283.

Kenakin, T., and Weiss, J. (1996). The evolution of drug-receptor models: The cubic ternary complex model for seven transmembrane domain receptors. *Biopharm. J.* **1,** 3–14.

Kim, J. Y., Borleis, J. A., and Devreotes, P. N. (1998). Switching of chemoattractant receptors programs development and morphogenesis in *Dictyostelium:* Receptor subtypes activate common responses at different agonist concentrations. *Dev. Biol.* **197,** 117–128.

Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., *et al.* (1992). Constitutive activation of the α_{1B}-adrenergic receptor by all amino acid substitutions at a single site. *J. Biol. Chem.* **267,** 1430–1433.

Kledal, T. N., Rosenkilde, M. M., Coulin, F., *et al.* (1997). A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science* **277,** 1656–1659.

Klein, P., Sun, T., Saxe, C., 3rd, *et al.* (1988). A chemoattractant receptor controls development in *Dictyostelium discoideum. Science* **241,** 1467–1472.

Kolakowski, L. F., Jr. (1994). GCRDb: A G-protein-coupled receptor database. *Receptors Channels* **2,** 1–7.

Kornau, H., Seeburg, P., and Kennedy, M. (1997). Interaction of ion channels and receptors with PDZ domain proteins. *Curr. Opin. Neurobiol.* **7,** 368–373.

Kostenis, E., Gomeza, J., Lerche, C., *et al.* (1997). Genetic analysis of receptor–Gαq coupling selectivity. *J. Biol. Chem.* **272,** 23675–23681.

Kozasa, T., Jiang, X., Hart, M. J., *et al.* (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science* **280,** 2109–2111.

Krasnoperov, V. G., Bittner, M. A., Beavis, R., *et al.* (1997). α-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. *Neuron* **18,** 925–937.

Kubo, Y., Miyashita, T., and Murata, Y. (1998). Structural basis for a Ca^{2+}-sensing function of the metabotropic glutamate receptors. *Science* **279,** 1722–1725.

Kuner, R., Kohr, G., Grunewald, S., *et al.* (1999). Role of heteromer formation in $GABA_B$ receptor function. *Science* **283,** 74–77.

Kunishima, N., Shimada, Y., and Tsuji, Y. *et al.* (2000). Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407,** 971–977.

Lambright, D. G., Noel, J. P., Hamm, H. E., *et al.* (1994). Structural determinants for activation of the α-subunit of a heterotrimeric G protein. *Nature* **369,** 621–628.

Lambright, D. G., Sondek, J., Bohn, A., *et al.* (1996). The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature* **379,** 311–319.

Laporte, S. A., Oakley, R. H., Zhang, J., *et al.* (1999). The beta2-adrenergic receptor/beta arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc. Natl. Acad. Sci. USA* **96,** 3712–3717.

Laporte, S. A., Oakley, R. H., Holt, J. A., *et al.* (2000). The interaction of beta-arrestin with the AP-2 adaptor is requird for the clustering of beta2-adrenergic receptor into clathrin-coated pits. *J. Biol. Chem.* **275,** 23120–23126.

Lazareno, S., Popham, A., and Birdsall, N. J. (2000). Allosteric interactions of staurosporine and other indolocarbazoles with N-[methyl-(3)H]scopolamine and acetylcholine at muscarinic receptor subtypes: Identification of a second allosteric site. *Mol. Pharmacol.* **58,** 194–207.

Lee, C., Murakami, T., and Simonds, W. F. (1995). Identification of a discrete region of the G protein γ subunit conferring selectivity in $\beta\gamma$ complex formation. *J. Biol. Chem.* **270,** 8779–8784.

Lee, D., George, S., Evans, J., *et al.* (2001). Orphan G protein-coupled receptors in the central nervous system. *Curr. Opin. Pharmacol.* **1,** 31–39.

Lefkowitz, R. J., Cotecchia, S., Samana, P., *et al.* (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.* **14,** 303–304.

Lezcano, N., Mrzljak, L., Eubanks, S., *et al.* (2000). Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein. *Science* **287,** 1660–1664.

Libert, F., Cochaux, P., Beckman, G., *et al.* (1998). The deltaccr5 mutation conferring protection against HIV-1 in Caucasian populations has a single and recent origin in northeastern Europe. *Hum. Mol. Genet.* **7,** 399–406.

Lin, L., Faraco, J., Li, R., *et al.* (1999). The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* **98,** 365–376.

Lin, S. W., and Sakmar, T. P. (1996). Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry* **35,** 11149–11159.

Lin, Y. J., Seroude, L., and Benzer, S. (1998). Extended life-span and stress resistance in the *Drosophila* mutant methuselah. *Science* **282,** 943–946.

Litschig, S., Gasparini, F., Rueegg, D., *et al.* (1998). CPCCOEt, a non-competitive mGluR1 antagonist, inhibits receptor signaling without affecting glutamate binding. *Mol. Pharmacol.* **55,** 453–461.

Liu, F., Wan, Q., Pristupa, Z. B., *et al.* (2000). Direct protein–protein coupling enables cross-talk between dopamine D5 and gamma-aminobutyric acid A receptors. *Nature* **403,** 274–280.

Liu, J., and Wess, J. (1996). Different single receptor domains determine the distinct G protein coupling profiles of members of the vasopressin receptor family. *J. Biol. Chem.* **271,** 8772–8778.

Liu, J., Conklin, B., Blin, N., *et al.* (1995). Identification of a receptor/G protein contact site critical for signaling specificity and G protein activation. *Proc. Natl. Acad. Sci. USA* **92,** 11642–11646.

Lopez, I., Mak, E. C., Ding, J., *et al.* (2001). A novel bifunctional phospholipase C that is regulated by G{alpha} 12 and stimulates the Ras/mitogen-activated protein kinase pathway. *J. Biol. Chem.* **276,** 2758–2765.

Luttrell, L. M., van Biesen, T., Hawes, B. E., *et al.* (1998). Regulation of mitogen-activated protein kinase pathways by catecholamine receptors. *Adv. Pharmacol.* **42,** 466–470.

Luttrell, L. M., Ferguson, S. S., Daaka, Y., *et al.* (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor–Src protein kinase complexes. *Science* **283,** 655–661.

MacEwan, D. J., and Milligan, G. (1996a). Inverse agonist-induced up-regulation of the human beta2-adrenoceptor in transfected neuroblastoma X glioma hybrid cells. *Mol. Pharmacol.* **50,** 1479–1486.

MacEwan, D. J., and Milligan, G. (1996b). Up-regulation of a constitutively active form of the beta2-adrenoceptor by sustained treatment with inverse agonists but not antagonists. *FEBS Lett.* **399,** 108–112.

MacEwan, D. J., Kim, G. D., and Milligan, G. (1996). Agonist regulation of adenylate cyclase activity in neuroblastoma × glioma hybrid NG108-15 cells transfected to co-express adenylate cyclase type II and the beta 2-adrenoceptor. Evidence that adenylate cyclase is the limiting component for receptor-mediated stimulation of adenylate cyclase activity. *Biochem. J.* **318,** 1033–1039.

Maggio, R., Vogel, Z., and Wess, J. (1993). Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. *Proc. Natl. Acad. Sci. USA* **90,** 3103–3107.

Maheshwari, H. G., Silverman, B. L., Dupuis, J., *et al.* (1998). Phenotype and genetic analysis of a syndrome caused by an inactivating mutation in the growth hormone-releasing hormone receptor: Dwarfism of Sindh. *J. Clin. Endocrinol. Metab.* **83,** 4065–4074.

Malitschek, B., Schweizer, C., Keir, M., *et al.* (1999). The N-terminal domain of gamma-aminobutyric acid(B) receptors is sufficient to specify agonist and antagonist binding. *Mol. Pharmacol.* **56,** 448–454.

Mancini, A., Koch, A., Stefan, M., *et al.* (2000). The direct association of the multiple PDZ domain containing proteins (MUPP-1) with the human c-Kit C-terminus is regulated by tyrosine kinase activity. *FEBS Lett.* **482,** 54–58.

Manivet, P., Mouillet-Richard, S., Callebert, J., *et al.* (2000). PDZ-dependent activation of nitric-oxide synthases by the serotonin 2B receptor. *J. Biol. Chem.* **275,** 9324–9331.

Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000). A trafficking checkpoint controls GABA(B) receptor heterodimerization. *Neuron* **27,** 97–106.

Marrero, M. B., Schieffer, B., Paxton, W. G., *et al.* (1995). Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature* **375,** 247–250.

Marrero, M. B., Venema, V. J., Ju, H., *et al.* (1999). Endothelial nitric oxide synthase interactions with G-protein-coupled receptors. *Biochem. J.* **343**(Pt. 2), 335–340.

Mary, S., Gomeza, J., Prézeau, L., *et al.* (1998). A cluster of basic residues in the carboxyl-terminal tail of the short metabotropic glutamate receptor 1 variants impairs their coupling to phospholipase C. *J. Biol. Chem.* **273,** 425–432.

Mattingly, R. R., and Macara, I. G. (1996). Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein beta gamma subunits. *Nature* **382,** 268–272.

Matus-Leibovitch, N., Nussensveig, D. R., Gershengorn, M. C., *et al.* (1995). Truncation of the thyrotropin-releasing hormone receptor carboxyl tail causes constitutive activity and leads to impaired responsiveness in *Xenopus* oocytes and AtT20 cells. *J. Biol. Chem.* **270,** 1041–1047.

Maximov, A., Sudhof, T. C., and Bezprozvanny, I. (1999). Association of neuronal calcium channels with modular adaptor proteins. *J. Biol. Chem.* **274,** 24453–24456.

Mazzoni, M. R., and Hamm, H. E. (1996). Interaction of transducin with light-activated rhodopsin protects it from proteolytic digestion by trypsin. *J. Biol. Chem.* **271,** 30034–30040.

McDonald, P. H., Cote, N. L., Lin, F. T., *et al.* (1999). Identification of NSF as a beta-arrestin 1-binding protein. Implications for beta2-adrenergic receptor regulation. *J. Biol. Chem.* **274,** 10677–10680.

Meunier, J. C., Mollereau, C., Toll, L., *et al.* (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **377,** 532–535.

Milligan, G. (1993). Agonist regulation of cellular G protein levels and distribution: Mechanisms and functional implications. *Trends Pharmacol. Sci.* **14,** 413–418.

Minakami, R., Jinnai, N., and Sugiyama, H. (1997). Phosphorylation and calmodulin binding of the metabotropic glutamate receptor subtype 5 (mGluR5) are antagonistic in vitro. *J. Biol. Chem.* **272,** 20291–20298.

Misrahi, M., Beau, I., Meduri, G., *et al.* (1998). Gonadotropin receptors and the control of gonadal steroidogenesis: Physiology and pathology. *Baillieres Clin. Endocrinol. Metab.* **12,** 35–66.

Mitchell, R., McCulloch, D., Lutz, E., *et al.* (1998). Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. *Nature* **392,** 411–414.

Mixon, M. B., Lee, E., Coleman, D. E., *et al.* (1995). Tertiary and quaternary structural changes in Giα1 induced by GTP hydrolysis. *Science* **270,** 954–960.

Mochizuki, N., Ohba, Y., Kiyokawa, E., *et al.* (1999). Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with G alpha(i). *Nature* **400,** 891–894.

Monnot, C., Bihoreau, C., Conchon, C., *et al.* (1996). Polar residues in the transmembrane domains of the type 1 angiotensin II receptor are required for binding and coupling. *J. Biol. Chem.* **271,** 1507–1513.

Monod, J., Wyman, J., and Changeux, J.-P. (1965). On the nature of allosteric transitions: A plausible model. *J. Mol. Biol.* **12,** 88–118.

Montell, C. (1997). New light on TRP and TRPL. *Mol. Pharmacol.* **52,** 755–763.

Morello, J. P., Salahpour, A., Laperriere, A., *et al.* (2000). Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J. Clin. Invest,* **105,** 887–895.

Morin, D., Cotte, N., Balestre, M.-N., *et al.* (1998). The D136A mutation of the V2 vasopressin receptor induces a constitutive activity which permits discrimination between antagonists with partial agonist and inverse agonists activities. *FEBS Lett.* **441,** 470–475.

Morisset, S., Traiffort, E., Arrang, J. M., *et al.* (2000). Changes in histamine H3 receptor responsiveness in mouse brain. *J. Neurochem.* **74,** 339–346.

Naisbitt, S., Kim, E., Tu, J. C., *et al.* (1999). Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. *Neuron* **23,** 569–582.

Nakajima, Y., Yamamoto, T., Nakayama, T., *et al.* (1999). A relationship between protein kinase C phosphorylation and calmodulin binding to the metabotropic glutamate receptor subtype 7. *J. Biol. Chem.* **274,** 27573–27577.

Nathans, J. (1999). The evolution and physiology of human color vision: Insights from molecular genetic studies of visual pigments. *Neuron* **24,** 299–312.

Natochin, M., McEntaffer, R. L., and Artemyev, N. O. (1998). Mutational analysis of the Asn residue essential for RGS protein binding to G-proteins. *J. Biol. Chem.* **273,** 6731–6735.

Naville, D., Barjhoux, L., Jaillard, C., *et al.* (1996). Mutations of ACTH receptor gene and familial syndrome of glucocorticoid deficiency. *Ann. Endocrinol.* **57,** 101–106.

Navon, S. E., and Fung, B. K. K. (1984). Characterization of transducin from bovine retinal rod outer segments: Mechanism and effects of cholera toxin-catalysed ADP ribosylation. *J. Biol. Chem.* **259,** 6686–6693.

Nehring, R. B., Horikawa, H. P., El Far, O., *et al.* (2000). The metabotropic GABAB receptor directly interacts with the activating transcription factor 4 (ATF-4). *J. Biol. Chem.* **275,** 35185–35191.

Nemeth, E. F., Steffey, M. E., Hammerland, L. G., *et al.* (1998). Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proc. Natl. Acad. Sci. USA* **95,** 4040–4045.

New, D., and Wong, J. (1998). The evidence for G-protein-coupled receptors and heterotrimeric G proteins in protozoa and ancestral metazoa. *Biol. Signals Receptors* **7,** 98–108.

Ng, G. Y., O'Dowd, B. F., Lee, S. P., *et al.* (1996). Dopamine D2 receptor dimers and receptor-blocking peptides. *Biochem. Biophys. Res. Commun.* **227,** 200–204.

Ng, G. Y., Clark, J., Coulombe, N., *et al.* (1999). Identification of a GABAB receptor subunit, gb2, required for functional GABAB receptor activity. *J. Biol. Chem.* **274,** 7607–7610.

Nicoletti, F., Wroblewski, J. T., Novelli, A., *et al.* (1986). The activation of inositol phospholipid metabolism as a signal-transduction system for excitatory amino acids in primary cultures of cerebellar granule cells. *J. Neurosci.* **6,** 1905–1911.

Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993). The 2.2 Å crystal structure of transducin-α complexed with GTPγS. *Nature* **366,** 654–663.

Northup, J. K., Smigel, M. D., Sternweis, P. C., *et al.* (1983). The subunits of the stimulatory regulatory component of adenylate cyclase. Resolution of the activated 45,000 Dalton (alpha) subunit. *J. Biol. Chem.* **258,** 11369–11376.

Oakley, R. H., Laporte, S. A., Holt, J. A., *et al.* (1999). Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J. Biol. Chem.* **274,** 32248–32257.

O'Brien, R. J., Lau, L. F., and Huganir, R. L. (1998). Molecular mechanisms of glutamate receptor clustering at excitatory synapses. *Curr. Opin. Neurobiol.* **8,** 364–369.

O'Connor, V., El Far, O., Bofill-Cardona, E., *et al.* (1999). Calmodulin dependence of presynaptic metabotropic glutamate receptor signaling. *Science* **286,** 1180–1184.

Offermanns, S., and Simon, M. I. (1995). Gα15 and Gα16 couple a wide variety of receptors to phospholipase C. *J. Biol. Chem.* **270,** 15175–15180.

O'Hara, P. J., Sheppard, P. O., Thogersen, H., *et al.* (1993). The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11,** 41–52.

Okamoto, T., Sekiyama, N., Otsu, M., *et al.* (1998). Expression and purification of extracellular ligand binding region of metabotropic glutamate receptor subtype. *J. Biol. Chem.* **273,** 13089–13096.

Okimura, J. T., and Norton, S. A. (1998). Jealousy and mutilation: Nose-biting as retribution for adultery. *Lancet* **352,** 2010–2011.

Oliviera, L., Paiva, A. C. M., Sander, C., *et al.* (1994). A common step for signal transduction in G protein-coupled receptors. *Trends Pharmacol. Sci.* **15,** 170–172.

Orly, J., and Schramm, M. (1976). Coupling of catecholamines receptor from one cell with adenylate cyclase from another cell by cell fusion. *J. Biol. Chem.* **73,** 4410–4414.

Overton, M. C., and Blumer, K. J. (2000). G-protein-coupled receptors function as oligomers in vivo. *Curr. Biol.* **10,** 341–344.

Pagano, A., Ruegg, D., Litschig, S., *et al.* (2000). The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-la-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J. Biol. Chem.* **275,** 33750–33758.

Page, R. D. (1996). TreeView: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12,** 357–358.

Palczewski, K., Kumasaka, T., Hori, T., *et al.* (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289,** 739–745.

Pantaloni, C., Brabet, P., Bilanges, B., *et al.* (1996). Alternative splicing in the N-terminal domain of the PACAP receptor modulates receptor selectivity and relative potencies of PACAP-27 and -38 in phospholipase C activation. *J. Biol. Chem.* **271,** 22146–22151.

Parker, E. M., and Ross, E. M. (1991). Truncation of the extended carboxyl-terminal domain increases the expression and regulatory activity of the avian β-adrenergic receptor. *J. Biol. Chem.* **266,** 9987–9996.

Parma, J., Duprez, L., Van Sande, J., *et al.* (1993). Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenoma. *Nature* **365,** 649–651.

Parma, J., Van Sande, J., Swillens, S., *et al.* (1995). Somatic mutations causing constitutive activity of the thyrotropin receptor are the major cause of hyperfunctioning thyroid adenomas: Identification of additional mutations activating both the cyclic adenosine 3′,5′-monophosphate and inositol phosphate-Ca^{2+} cascades. *Mol. Endocrinol.* **9,** 725–733.

Parmentier, M. L., Pin, J. P., Bockaert, J., *et al.* (1996). Cloning and functional expression of a *Drosophila* metabotropic glutamate receptor expressed in the embryonic CNS. *J. Neurosci.* **16,** 6687–6694.

Parmentier, M.-L., Joly, C., Restituito, S., *et al.* (1998). The G-protein coupling profile of the metabotropic glutamate receptors, as determined with exogenous proteins, is independent of their ligand recognition domain. *Mol. Pharmacol.* **53,** 778–786.

Parmentier, M. L., Galvez, T., Acher, F., *et al.* (2000a). Conservation of the ligand recognition site of metabotropic glutamate receptors during evolution. *Neuropharmacology* **39,** 1119–1131.

Parmentier, M. L., Woods, D., Greig, S., *et al.* (2000b). Rapsynoid, partner of Inscuteable, controls asymmetric division of larval neuroblasts in *Drosophila. J. Neurosci.* **20,** RC84 1.

Patapoutian, A., and Reichardt, L. F. (2000). Roles of Wnt proteins in neural development and maintenance. *Curr. Opin. Neurobiol.* **10,** 392–399.

Perrimon, N. (1996). Serpentine proteins slither into the wingless and hedgehog fields. *Cell* **86,** 513–516.

Peyron, C., Faraco, J., Rogers, W., *et al.* (2000). A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nature Med.* **6,** 991–997.

Phang, T., Kundu, G., Hong, S., *et al.* (1998). The amino-terminal region of the luteinizing hormone/choriogonadotropin receptor contacts both subunits of human choriogonadotropin. II. Photoaffinity labeling. *J. Biol. Chem.* **273,** 13841–13847.

Pin, J. P., and Duvoisin, R. (1995). The metabotropic glutamate receptors: Structure and functions. *Neuropharmacology* **34,** 1–26.

Pin, J. P., De Colle, C., Bessis, A. S., *et al.* (1999). New perspectives for the development of selective metabotropic glutamate receptor ligands. *Eur. J. Pharmacol.* **375,** 277–294.

Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998). G protein-coupled receptor kinases. *Annu. Rev. Biochem.* **67,** 653–692.

Plakidou-Dymock, S., Dymock, D., and Hooley, R. (1998). A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. *Curr. Biol.* **8,** 315–324.

Porter, J. E., and Perez, D. M. (1999). Characteristics for a salt-bridge switch mutation of the alpha(1b) adrenergic receptor. Altered pharmacology and rescue of constitutive activity. *J. Biol. Chem.* **274,** 34535–34538.

Prehoda, K. E., Lee, D. J., and Lim, W. A. (1999). Structure of the enabled/VASP homology 1 domain–peptide complex: A key component in the spatial control of actin assembly. *Cell* **97,** 471–480.

Prézeau, L., Gomeza, J., Ahern, S., *et al.* (1996). Changes in the carboxyl-terminal domain of metabotropic glutamate receptor 1 by alternative splicing generate receptors with differing agonist-independent activity. *Mol. Pharmacol.* **49,** 422–429.

Prézeau, L., Richman, J. G., Edwards, S. W., *et al.* (1999). The zeta isoform of 14-3-3 proteins interacts with the third intracellular loop of different alpha2-adrenergic receptor subtypes. *J. Biol. Chem.* **274,** 13462–13469.

Puffenberger, E. G., Hosoda, K., Washington, S. S., *et al.* (1994). A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* **79,** 1257–1266.

Quitterer, U., Zaki, E., and AbdAlla, S. (1999). Investigation of the extracellular accessibility of the connecting loop between membrane domains I and II of the bradykinin B2 receptor. *J. Biol. Chem.* **274,** 14773–14778.

Rasmussen, S. G., Jensen, A. D., Liapakis, G., *et al.* (1999). Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: Constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol. Pharmacol.* **56,** 175–184.

Rath, P., DeCaluwe, L. L., Bovee-Geurts, P. H., *et al.* (1993). Fourier transform infrared difference spectroscopy of rhodopsin mutants: Light activation of rhodopsin causes hydrogen-bonding change in residue aspartic acid-83 during meta II formation. *Biochemistry* **32,** 10277–102782.

Reinscheid, R. K., Nothacker, H. P., Bourson, A., *et al.* (1995). Orphanin FQ: A neuropeptide that activates an opioid-like G protein-coupled receptor. *Science* **270,** 792–794.

Robbins, L. S., Nadeau, J. H., Johnson, K. R., *et al.* (1993). Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell* **72,** 827–834.

Robbins, M. J., Ciruela, F., Rhodes, A., *et al.* (1999). Characterization of the dimerization of metabotropic glutamate receptors using an N-terminal truncation of mGluR 1 alpha. *J. Neurochem.* **72,** 2539–2547.

Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., *et al.* (1992). Constitutively active mutants of rhodopsin. *Neuron* **9,** 719–725.

Rocheville, M., Lange, D. C., Kumar, U., *et al.* (2000a). Receptors for dopamine and somatostatin: Formation of hetero-oligomers with enhanced functional activity. *Science* **288,** 154–157.

Rocheville, M., Lange, D. C., Kumar, U., *et al.* (2000b). Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J. Biol. Chem.* **275,** 7862–7869.

Rodbell, M. (1992). The role of GTP-binding proteins in signal transduction: From the sublimely simple to the conceptually complex. *Curr. Topics Cell. Regul.* **32,** 1–47.

Rodbell, M. (1995). Nobel lecture. Signal transduction: Evolution of an idea. *Biosci. Rep.* **15,** 117–133.

Rodbell, M., Birnbaumer, L., Pohl, S. L., *et al.* (1971). The glucagon-sensitive adenylyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. *J. Biol. Chem.* **246,** 1877–1882.

Rodien, P., Bremont, C., Sanson, M. L., *et al.* (1998). Familial gestational hyperthyroidism caused by a mutant thyrotropin receptor hypersensitive to human chorionic gonadotropin. *N. Engl. J. Med.* **339,** 1823–1826.

Romano, C., Yang, W.-L., and O'Malley, K. L. (1996). Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J. Biol. Chem.* **271,** 28612–28616.

Rosenfeld, P. J., Cowley, G. S., McGee, T. L., *et al.* (1992). A null mutation in the rhodopsin gene causes rod photoreceptor dysfunction and autosomal recessive retinitis pigmentosa. *Nat. Genet.* **1,** 209–213.

Rosenkilde, M. M., Kledal, T. N., Brauner-Osborne, H., *et al.* (1999). Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74. *J. Biol. Chem.* **274,** 956–961.

Roush, W. (1996). Regulating G protein signaling [news]. *Science* **271,** 1056–1058.

Rubin, G. M., Yandell, M. D., Wortman, J. R., *et al.* (2000). Comparative genomics of the eukaryotes. *Science* **287,** 2204–2215.

Ruiz i Altaba, R. (1997). Catching a Gli-mpse of hedgehog. *Cell* **90,** 193–196.

Sabroe, I., Peck, M. J., Van Keulen, B. J., *et al.* (2000). A small molecule antagonist of chemokine receptors CCR1 and CCR3. Potent inhibition of eosinophil function and CCR3-mediated HIV-1 entry. *J. Biol. Chem.* **275,** 25985–25992.

Saito, Y., Nothacker, H. P., Wang, Z., *et al.* (1999). Molecular characterization of the melanin-concentrating-hormone receptor. *Nature* **400,** 265–269.

Sakurai, T., Amemiya, A., Ishii, M., *et al.* (1998). Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* **92,** 573–585.

Salahpour, A., Angers, S., and Bouvier, M. (2000). Functional significance of oligomerization of G-protein-coupled receptors. *Trends Endocrinol Metab.* **11,** 163–168.

Salvatori, R., Hayashida, C. Y., Aguiar-Oliveira, M. H., *et al.* (1999). Familial dwarfism due to a novel mutation of the growth hormone-releasing hormone receptor gene. *J. Clin. Endocrinol. Metab.* **84,** 917–923.

Samama, P., Cotecchia, S., Costa, T., *et al.* (1993). A mutation-induced activated state of the β_2-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* **268,** 4625–4635.

Samson, M., Libert, F., Doranz, B. J., *et al.* (1996). Resistance to HIV-1 infection of Caucasian individuals bearing mutant alleles of the CCR5 chemokine receptor gene. *Nature* **382,** 722–725.

Sanchez, J. E., Perera, E., Baumbach, L., *et al.* (1998). Growth hormone receptor mutations in children with idiopathic short stature. *J. Clin. Endocrinol. Metab.* **83,** 4079–4083.

Scheer, A., Fanelli, F., Costa, T., *et al.* (1996). Constitutively active mutants of the α_{1B}-adrenergic receptor: Role of highly conserved polar amino acids in receptor activation. *EMBO J.* **15,** 3566–3578.

Scheer, A., Fanelli, F., Costa, T., *et al.* (1997). The activation process of the alpha 1B-adrenergic receptor: Potential role of protonation and hydrophobicity of a highly conserved aspartate. *Proc. Natl. Acad. Sci. USA* **94,** 808–813.

Scheer, A., Costa, T., Fanelli, F., *et al.* (2000). Mutational analysis of the highly conserved arginine within the Glu/Asp–Arg–Tyr motif of the alpha (1b)-adrenergic receptor: Effects on receptor isomerization and activation. *Mol. Pharmacol.* **57,** 219–231.

Schiff, M. L., Siderovski, D. P., Jordan, J. D., *et al.* (2000). Tyrosine-kinase-dependent recruitment of RGS12 to the N-type calcium channel. *Nature* **408,** 723–727.

Schipani, E., Kruse, K., and Juppar, H. (1995). A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science* **268,** 98–100.

Schipani, E., Langman, C. B., Parfitt, A. M., *et al.* (1996). Constitutively activated receptors for parathyroid hormone and parathyroid hormone-related peptide in Jansen's metaphyseal chondrodysplasia. *N. Engl. J. Med.* **335,** 708–714.

Schlegel, W., Kempner, E. S., and Rodbell, M. (1979). Activation of adenylate cyclase in hepatic membranes involves interactions of the catalytic unit with multimeric complexes of regulatory proteins. *J. Biol. Chem.* **254,** 5168–5176.

Schmitt, J. M., and Stork, P. J. (2000). Beta 2-adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. *J. Biol. Chem.* **275,** 25342–25350.

Schoneberg, T., Yun, J., Wenkert, D., *et al.* (1996). Functional rescue of mutant V2 vasopressin receptors causing nephrogenic diabetes insipidus by a co-expressed receptor polypetide. *EMBO J.* **15,** 1283–1291.

Schoneberg, T., Sandig, V., Wess, J., *et al.* (1997). Reconstitution of mutant V2 vasopressin receptors by adenovirus-mediated gene transfer. Molecular basis and clinical implication. *J. Clin. Invest.* **100,** 1547–1556.

Schulz, A., Grosse, R., Schultz, G., *et al.* (2000). Structural implication for receptor oligomerization from functional reconstitution studies of mutant V2 vasopressin receptors. *J. Biol. Chem.* **275,** 2381–2389.

Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., *et al.* (1996). Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. *Nature* **383,** 347–350.

Sheikh, S. P., Vilardarga, J. P., Baranski, T. J., *et al.* (1999). Similar structures and shared switch mechanisms of the beta2-adrenoceptor and the parathyroid hormone receptor. Zn(II) bridges between helices III and VI block activation. *J. Biol. Chem.* **274,** 17033–17041.

Shenker, A., Laue, L., Kosugi, S., *et al.* (1993). A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* **365,** 652–654.

Shichida, Y., and Imai, H. (1998). Visual pigment: G-protein-coupled receptor for light signals. *Cell. Mol. Life Sci.* **54,** 1299–1315.

Shields, J. E., Dinovo, E. C., Henriksen, R. A., *et al.* (1967). The purification and amino acid composition of bovine rhodopsin. *Biochim. Biophys. Acta* **147,** 238–251.

Shiraishi, Y., Mizutani, A., Bito, H., *et al.* (1999). Cupidin, an isoform of Homer/Vesl, interacts with the actin cytoskeleton and activated rho family small GTPases and is expressed in developing mouse cerebellar granule cells. *J. Neurosci.* **19,** 8389–8400.

Shorr, R. G., Lefkowitz, R. J., and Caron, M. G. (1981). Purification of the beta-adrenergic receptor. Identification of the hormone binding subunit. *J. Biol. Chem.* **256,** 5820–5826.

Siderovski, D. P., Diverse-Pierluissi, M., and De Vries, L. (1999). The GoLoco motif: A Galphai/o binding motif and potential guanine-nucleotide exchange factor. *Trends Biochem. Sci.* **24,** 340–341.

Simon, M. N., De Virgilio, C., Souza, B., *et al.* (1995). Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway. *Nature* **376,** 702–705.

Sladeczek, F., Pin, J.-P., Récasens, M., *et al.* (1985). Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature* **317,** 717–719.

Smith, F. D., Oxford, G. S., and Milgram, S. L. (1999). Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J. Biol. Chem.* **274,** 19894–19900.

Snow, B. E., Hall, R. A., Krumins, A. M., *et al.* (1998). GTPase activating specificity of RGS12 and binding specificity of an alternatively spliced PDZ (PSD-95/Dlg/ZO-1) domain. *J. Biol. Chem.* **273,** 17749–17755.

Sondek, J., Lambright, D. G., Noel, J. P., *et al.* (1994). GTPase mechanism of G proteins from 1.7 Å crystal structure of transducin α-GDP. AlF_4. *Nature* **372,** 276–279.

Songyang, Z., Fanning, A. S., Fu, C., *et al.* (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* **275,** 73–77.

Speca, D. J., Lin, D. M., Sorensen, P. W., *et al.* (1999). Functional identification of a goldfish odorant receptor. *Neuron* **23,** 487–498.

Spengler, D., Waeber, C., Pantaloni, C., *et al.* (1993). Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365,** 170–175.

Spiegel, A. M. (1996). Defects in G protein-coupled signal transduction in human disease. *Annu. Rev. Physiol.* **58,** 143–170.

Spiegel, A. M., Weinstein, L. S., and Shenker, A. (1993). Abnormalities in G protein-coupled signal transduction pathways in human disease. *J. Clin. Invest.* **92,** 1119–1125.

Sprang, S. R. (1997). G protein mechanisms: Insights from structural analysis. *Annu. Rev. Biochem.* **66,** 639–678.

Stacey, M., Lin, H. H., Gordon, S., *et al.* (2000). LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. *Trends Biochem. Sci.* **25,** 284–289.

Stadel, J. M., Wilson, S., and Bergsam, D. J. (1997). Orphan G protein-coupled receptors: A neglected opportunity for pioneer drug discovery. *Trends Pharmacol. Sci.* **18,** 430–437.

Strosberg, A. D. (1997). Association of beta 3-adrenoceptor polymorphism with obesity and diabetes: Current status. *Trends Pharmacol. Sci.* **18,** 449–454.

Sudol, M. (1998). From Scr homology domains to other signaling modules: Proposal of the "protein recognition code." *Oncogene* **17,** 1469–1474.

Sunahara, R. K., Tesmer, J. J., Gilman, A. G., *et al.* (1997). Crystal structure of the adenylyl cyclase activator Gsalpha. *Science* **278,** 1943–1947.

Sung, C. H., Makino, C., Baylor, D., *et al.* (1994). A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. *J. Neurosci.* **14,** 5818–5833.

Sutherland, E. W., and Rall, T. W. (1958). Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue. *J. Biol. Chem* **232,** 1077–1091.

Swanson, J., Oosterlaan, J., Murias, M., *et al.* (2000). Attention deficit/hyperactivity disorder children with a 7-repeat allele of the dopamine receptor D4 gene have extreme behavior but normal performance on critical neuropsychological tests of attention. *Proc. Natl. Acad. Sci. USA* **97,** 4754–4759.

Tadokoro, S., Tachibana, T., Imanaka, T., *et al.* (1999). Involvement of unique leucine-zipper motif of PSD-Zip45 (Homer lc/vesl-1L) in group 1 metabotropic glutamate receptor clustering. *Proc. Natl. Acad. Sci. USA* **96,** 13801–13806.

Tai, A. W., Chuang, J. Z., Bode, C., *et al.* (1999). Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell* **97,** 877–887.

Takeda, H., Matozaki, T., Takada, T., *et al.* (1999). PI 3-kinase gamma and protein kinase C-zeta mediate RAS-independent activation of MAP kinase by a Gi protein-coupled receptor. *EMBO J.* **18,** 386–395.

Talbodec, A., Berkane, N., Blandin, V., *et al.* (2000). Aspirin and sodium salicylate inhibit endothelin ETA receptors by an allosteric type of mechanism. *Mol. Pharmacol.* **57,** 797–804.

Tang, W., and Gilman, A. G. (1991). Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science* **254,** 1500–1503.

Tarasova, N. I., Rice, W. G., and Michejda, C. J. (1999). Inhibition of G-protein-coupled receptor function by disruption of transmembrane domain interactions. *J. Biol. Chem.* **274,** 34911–34915.

Tatemoto, K., Hosoya, M., Habata, Y., *et al.* (1998). Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.* **251,** 471–476.

Taylor, J. M., Jacob-Mosier, G. G., Lawton, R., *et al.* (1994). Binding of an α_2 adrenergic receptor third intracellular loop peptide to Gβ and the amino terminus of Gα. *J. Biol. Chem.* **269,** 27618–27624.

Taylor, J. M., Jacob-Mosier, G. G., Lawton, R., *et al.* (1996). Receptor and membrane interaction sites on Gβ. A receptor-derived peptide binds to the carboxyl terminus. *J. Biol. Chem.* **271,** 3336–3339.

Tesmer, J. G., Berman, D. M., Gilman, A. G., *et al.* (1997a). Structure of RGS4 bound to AIF4-activated Giα1: Stabilization of the transition state for GTP hydrolysis. *Cell* **89,** 251–261.

Tesmer, J. G., Sunahara, R. K., Gilman, A. G., *et al.* (1997b). Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsα GTPγS. *Science* **278,** 1907–1916.

Thannickal, T. C., Moore, R. Y., Nienhuis, R., *et al.* (2000). Reduced number of hypocretin neurons in human narcolepsy. *Neuron* **27,** 469–474.

Themmen, A. P., Martens, J. W., and Brunner, H. G. (1997). Gonadotropin receptor mutations. *J. Endocrinol.* **153,** 179–183.

Thompson, J. D., Gibson, T. J., Plewniak, F., *et al.* (1997). The CLUSTAL:X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25,** 4876–4882.

Tota, M. R., and Strader, C. D. (1990). Characterization of the binding domain of β-adrenergic receptor with the fluorescent antagonist carazolol. Evidence for a buried ligand binding site. *J. Biol. Chem.* **26,** 16891–16897.

Tsang, S. H., Burns, M. E., Calvert, P. D., *et al.* (1998). Role for the target enzyme in deactivation of photoreceptor G protein in vivo. *Science* **282,** 117–121.

Tsao, P., and Zastrow, M. (2000). Down-regulation of G protein-coupled receptors. *Curr. Opin. Neurobiol.* **10,** 365–369.

Tsigos, C., Arai, K., Hung, W., *et al.* (1993). Hereditary isolated glucocorticoid deficiency is associated with abnormalities of the adrenocorticotropin receptor gene. *J. Clin. Invest.* **92,** 2458–2461.

Tsuji, Y., Shimada, Y., Takeshita, T., *et al.* (2000). Cryptic dimer interface and domain organization of the extracellular region of metabotropic glutamate receptor subtype 1. *J. Biol. Chem.* **275,** 28144–28151.

Tsunoda, S., Sierralta, J., Sun, Y., *et al.* (1997). A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature* **388,** 243–249.

Tu, J. C., Xiao, B., Yuan, J. P., *et al.* (1998). Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron* **21,** 717–726.

Tu, J. C., Xiao, B., Naisbitt, S., *et al.* (1999). Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* **23,** 583–592.

Ullmer, C., Schmuck, K., Figge, A., *et al.* (1998). Cloning and characterization of MUPP1, a novel PDZ domain protein. *FEBS Lett.* **424,** 63–68.

Umemori, H., Inoue, T., Kume, S., *et al.* (1997). Activation of the G protein Gq/11 through tyrosine phosphorylation of the alpha subunit. *Science* **276,** 1878–1881.

Unger, V. M., Hargrave, P. M., Baldwin, J. M., *et al.* (1997). Arrangement of rhodopsin transmembrane alpha-helices. *Nature* **389,** 203–206.

Usiello, A., Baik, J.-H., Rougé-Pont, F., *et al.* (2000). Distinct functions of the two isoforms of dopamine D2 receptors. *Nature* **408,** 199–203.

Vaisse, C., Clement, K., Guy-Grand, B., *et al.* (1998). A frameshift mutation in human MC4R is associated with a dominant form of obesity [letter]. *Nat. Genet.* **20,** 113–114.

Van Sande, J., Parma, J., Tonacchera, M., *et al.* (1995). Somatic and germline mutations of the TSH receptor gene in thyroid diseases. *J. Clin. Endocrinol. Metab.* **80,** 2577–2585.

Varrault, A., Bockaert, J., and Waeber, C. (1992). Activation of 5-HT_{1A} receptors expressed in NIH-3T3 cells induces focus formation and potentiates EGF effect on DNA synthesis. *Mol. Biol. Cell* **3,** 961–969.

Venema, R. C., Ju, H., Venema, V. J., *et al.* (1998). Angiotensin II-induced association of phospholipase Cgamma1 with the G-protein-coupled AT1 receptor. *J. Biol. Chem.* **273,** 7703–7708.

Vernier, P., Cardinaud, B., Valdenaire, O., *et al.* (1995). An evolutionary view of drug–receptor interaction: The bioamine receptor family. *Trends Pharmacol. Sci.* **16,** 375–381.

Vila-Coro, A. J., Rodriguez-Frade, J. M., Martin De Ana, A., *et al.* (1999). The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J.* **13,** 1699–1710.

Vila-Coro, A. J., Mellado, M., Martin de Ana, A., *et al.* (2000). HIV-1 infection through the CCR5 receptor is blocked by receptor dimerization. *Proc. Natl. Acad. Sci. USA* **97,** 3388–3393.

Vosshall, L. B., Amrein, H., Morozov, P. S., *et al.* (1999). A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* **96,** 725–736.

Vossler, M. R., Yao, H., York, R. D., *et al.* (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89,** 73–82.

Vuong, T. M., and Chabre, M. (1990). Subsecond deactivation of transducin by endogenous GTP hydrolysis. *Nature* **346,** 71–74.

Wajnrajch, M. P., Gertner, J. M., Harbison, M. D., *et al.* (1996). Nonsense mutation in the human growth hormone-releasing hormone receptor causes growth failure analogous to the little (lit) mouse. *Nat. Genet.* **12,** 88–90.

Wall, M. A., Coleman, D. E., Lee, E., *et al.* (1995). The structure of the G protein heterotrimer. *Cell* **83,** 1047–1058.

Wedegaertner, P. B., and Bourne, H. (1994). Activation and depalmitoylation of Gsα. *Cell* **77,** 1062–1070.

Weiner, N. (1961). *Cybernetics: Or Control and Communication in the Animal and the Machine.* MIT Press, Cambridge, MA.

Weiss, E. R., Osawa, S., Shi, W., *et al.* (1994). Effects of carboxyl-terminal truncation on the stability and G protein-coupling activity of bovine rhodopsin. *Biochemistry* **33,** 7587–7593.

Wells, T. N., and Schwartz, T. W. (1997). Plagiarism of the host immune system: Lessons about chemokine immunology from viruses. *Curr. Opin. Biotechnol.* **8,** 741–748.

Wess, J. (1997). G-protein-coupled receptors: Molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J.* **11,** 346–354.

White, J. H., Wise, A., Main, M. J., *et al.* (1998). Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature* **396,** 679–682.

White, J., McIllhinney, R., Wise, A., *et al.* (2000). The $GABA_B$ receptor interacts directly with the related transcription factors CREB2 and ATFx. *Proc. Natl. Acad. Sci. USA* **97,** 13967–13972.

Wieland, T., and Chen, C. K. (1999). Regulators of G-protein signalling: A novel protein family involved in timely deactivation and desensitization of signalling via heterotrimeric G proteins. *Naunyn Schmiedebergs Arch. Pharmacol.* **360,** 14–26.

Wilson, S., Bergsma, D. J., Chambers, J. K., *et al.* (1998). Orphan G-protein-coupled receptors: The next generation of drug targets?. *Br. J. Pharmacol.* **125,** 1387–1392.

Wojcik, J., Berg, M. A., Esposito, N., *et al.* (1998). Four contiguous amino acid substitutions, identified in patients with Laron syndrome, differently affect the binding affinity and intracellular trafficking of the growth hormone receptor. *J. Clin. Endocrinol. Metab.* **83,** 4481–4489.

Wreggett, K. A., and De Lean, A. (1984). The ternary complex model. Its properties and application to ligand interactions with the D2-dopamine receptor of the anterior pituitary gland. *Mol. Pharmacol.* **26,** 214–227.

Xiao, B., Tu, J. C., Petralia, R. S., *et al.* (1998). Homer regulates the association of group 1 metabotropic receptors with multivalent complexes of Homer-related, synaptic proteins. *Neuron* **21,** 701–716.

Xiao, B., Tu, J. C., and Worley, P. F. (2000). Homer: A link between neural activity and glutamate receptor function. *Curr. Opin. Neurobiol.* **10,** 370–374.

Xu, N., Voyno-Yasenetskaya, T., and Gutkind, J. S. (1994). Potent transforming activity of the G13 alpha subunit defines a novel family of oncogenes. *Biochem. Biophys. Res. Commun.* **201,** 603–609.

Xu, X. Z., Choudhury, A., Li, X., *et al.* (1998). Coordination of an array of signaling proteins through homo- and heteromeric interactions between PDZ domains and target proteins. *J. Cell Biol.* **142,** 545–555.

Xu, Y. K., and Nusse, R. (1998). The Frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases [letter]. *Curr. Biol.* **8,** R405–R406.

Yamashita, S., Mochizuki, N., Ohba, Y., *et al.* (2000). CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *J. Biol. Chem.* **275,** 25488–25493.

Yeo, G. S., Farooqi, I. S., Aminian, S., *et al.* (1998). A frameshift mutation in MC4R associated with dominantly inherited human obesity [letter]. *Nat. Genet.* **20,** 111–112.

Yokoyama, S., Zhang, H., Radlwimmer, F. B., *et al.* (1999). Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). *Proc. Natl. Acad. Sci. USA* **96,** 6279–6284.

Zeng, F. Y., and Wess, J. (1999). Identification and molecular characterization of m3 muscarinic receptor dimers. *J. Biol. Chem.* **274,** 19487–19497.

Zhang, P., Jobert, A. S., Couvineau, A., *et al.* (1998). A homozygous inactivating mutation in the parathyroid hormone/parathyroid hormone-related peptide receptor causing *Blomstrand chondrodysplasia. J. Clin. Endocrinol. Metab.* **83,** 3365–3368.

Zhou, W., Flanagan, C., Ballesteros, J. A., *et al.* (1994). A reciprocal mutation supports helix 2 and helix 7 proximity in gonodotropin-releasing hormone receptor. *Mol. Pharmacol.* **45,** 165–170.

Zhou, X.-M., and Fishman, P. H. (1991). Desensitization of the human β_1-adrenergic receptor. *J. Biol. Chem.* **266,** 7462–7468.

Zhu, X., and Wess, J. (1998). Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* **37,** 15773–15784.

Zitzer, H., Honck, H. H., Bachner, D., *et al.* (1999a). Somatostatin receptor interacting protein defines a novel family of multidomain proteins present in human and rodent brain. *J. Biol. Chem.* **274,** 32997–33001.

Zitzer, H., Richter, D., and Kreienkamp, H. J. (1999b). Agonist-dependent interaction of the rat somatostatin receptor subtype 2 with cortactin-binding protein 1. *J. Biol. Chem.* **274,** 18153–18156.

Zwick, E., Hackel, P. O., Prenzel, N., *et al.* (1999). The EGF receptor as central transducer of heterologous signalling systems. *Trends Pharmacol. Sci.* **20,** 408–412.

Genes Involved in the Initiation of DNA Replication in Yeast

Arturo Calzada and Avelino Bueno
Instituto de Microbiología—Bioquímica/Centro de Investigación del Cáncer, Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno, CSIC/Universidad de Salamanca, 37007 Salamanca, Spain

Replication and segregation of the information contained in genomic DNA are strictly regulated processes that eukaryotic cells alternate to divide successfully. Experimental work on yeast has suggested that this alternation is achieved through oscillations in the activity of a serine/threonine kinase complex, CDK, which ensures the timely activation of DNA synthesis. At the same time, this CDK-mediated activation sets up the basis of the mechanism that ensures ploidy maintenance in eukaryotes. DNA synthesis is initiated at discrete sites of the genome called origins of replication on which a prereplicative complex (pre-RC) of different protein subunits is formed during the G_1 phase of the cell division cycle. Only after pre-RCs are formed is the genome competent to be replicated. Several lines of evidence suggest that CDK activity prevents the assembly of pre-RCs ensuring single rounds of genome replication during each cell division cycle. This review offers a descriptive discussion of the main molecular events that a unicellular eukaryote such as the budding yeast *Saccharomyces cerevisiae* undergoes to initiate DNA replication.

KEY WORDS: Cell cycle, Replication origins, Prereplicative complex, Postreplicative complex, DNA replication initiators, Regulation of DNA replication, Ploidy maintenance.

I. Introduction

The molecular events involved in regulating the initiation of DNA replication have been the subject of intensive investigation during the past two decades, in particular

in unicellular eukaryotes such as yeast. A direct consequence of this development in yeast molecular and cellular biology is that most of the elements involved in controls that regulate the initiation of DNA replication have been identified and we are beginning to understand how initiation of DNA synthesis works in these simple eukaryotes. We discuss all aspects related to the initiation events. With this purpose, we first describe how the cell cycle machinery is regulated to establish a G_1 period, characterized by a low cyclin-dependent kinase (CDK) activity, required both for the expression of genes involved in DNA synthesis and for the assembly of the complexes needed to activate origins of replication. We next describe different elements and proteins forming these complexes [prereplicative complexes or (pre-RCs)] and major lines of evidence supporting their role in DNA synthesis initiation. We also include a description of yeast replication origins, protein components of pre-RCs, and regulators involved in their activation. In Section IV we describe the current understanding of how cells establish a state of competence for replication at origins in a sequential/hierarchical process, from the end of mitosis to the G_1 to S phase transition, that eventually results in CDK-mediated activation of DNA synthesis. Section V explains how the regulatory action of kinases (CDKs) in DNA synthesis initiation integrates both the synchronic activation of multiple origins of replication and the prevention of an extra round of DNA replication within the same cell division cycle.

Although there are excellent reviews concerning specific aspects of this issue, we have tried to present a (different) functional description of genes involved in the initiation of DNA replication instead of presenting a gene-by-gene summary because we believe that the result affords a comprehensive way to understand how cells organize and activate the processes that lead to genome duplication. Although the long-review format of *International Review of Cytology* allows us to put forward an exhaustive summary of current knowledge about this topic, we have set limits to several aspects and must therefore apologize to many scientists that we may have failed to mention in this review.

II. Eukaryotic Cell Cycle

Genome transfer from parents to progeny is one of the most accurately controlled processes in the cell division cycle since the genetic information contained in the genome of all living organisms must be duplicated and transmitted faithfully from mother to daughter cells. On studying problems related to cell division and cancer, it is crucial to understand the mechanisms by which cells regulate the initiation of DNA replication. How do cells drive the replication of their DNA? Studies carried out during the past 15 years suggest that the same kinase activity that controls cell cycle progression also activates and inhibits the initiation of DNA replication. This apparent paradox would ensure that genome replication takes place only once for each cell cycle.

A. Eukaryotic Cell Division Cycle

One of the most significant inferences of cell theory, first suggested by Matthias Schleiden, working with plants in 1838, and extended to animals by Theodor Schwann 1 year later, is that cells only come from the division of a (pre-)existent mother cell. This principle regarding the generality of cellular organization in all living organisms establishes the basis of cell proliferation and cell division. The joint, hierarchical processes that result in the duplication of all cell components followed by the appropriate segregation into two daughter cells are known as the cell division cycle. Studies begun in the late 1970s have provided us with the understanding that cell division is primarily controlled by the molecular regulation of the timing of the initiation of DNA replication and the initiation of mitosis.

The essential mechanisms regulating genome replication and nuclear and cell division are similar in all eukaryotic cells, although details at the molecular level may differ between organisms. Because of this similarity, cell cycle research with fission and budding yeast has made significant contributions to the understanding of the controls regulating the molecular events involved in cell division. Classically divided into two periods—interphase and mitosis—four different phases distinguish between early and late stages in the cell cycle interval (Fig. 1). This

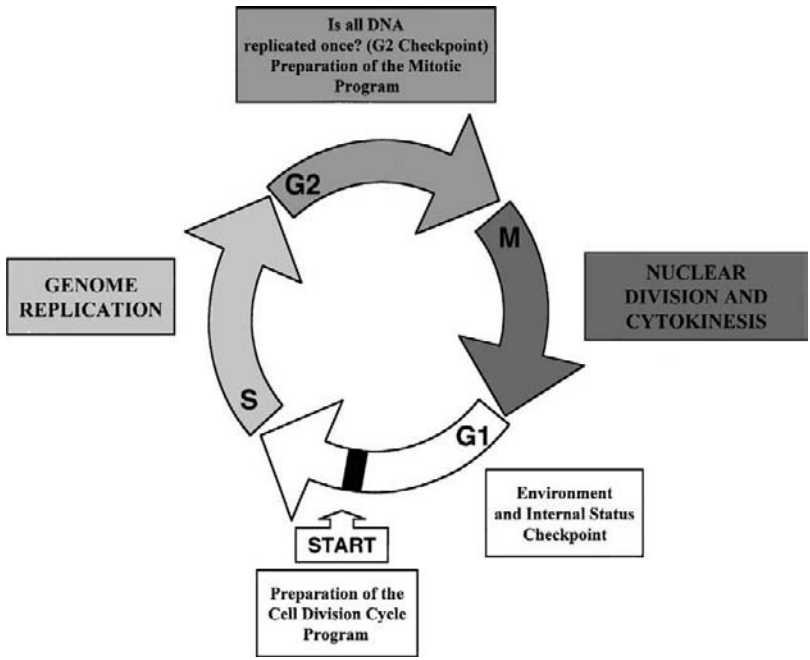


FIG. 1 The eukaryotic cell division cycle. Schematic drawing of the different phases in a cell division cycle of exponentially growing cells.

"landmark" of G_1 is called START in yeast and "restriction point" in multicellular systems, and it defines a point after which cells are committed to undergoing a complete round of cell division (Hartwell *et al.,* 1974; Johnston, 1977; Lew and Reed, 1992). During the G_2 interval of the division cycle, the cell analyzes whether DNA replication has been completed successfully and, if this is not the case, provides the time and mechanisms to repair possible DNA damage or to complete an incomplete genome replication. Under these conditions, or under normal conditions in which no repair or synthesis are needed, the G_2 phase prepares the cells for the segregation program that begins during mitosis.

It is clear that yeasts (Fig. 2; see color insert) can serve as a model for studies in cell division cycle regulation, although there are some peculiarities such as the already mentioned lack of nuclear envelope breakdown or the low degree of chromosomal condensation, if any, particularly in *Saccharomyces cerevisiae*. In contrast, yeasts offer many advantages that have been essential for the understanding of cell division cycle regulation. One of these advantages is the generation time: rapidly growing *S. cerevisiae* cells complete a cell division cycle in 90 min, in contrast to the 24-h period in rapidly replicating human cells in culture. Regardless of the generation time, perhaps the major breakthrough that yeasts have provided for cell division cycle analysis, and indeed in many other fields, is the genetic approach. Many studies and advances in knowledge of the cell cycle have occurred due to the isolation of mutants in specific genes related to cell division cycle control (*cdc* mutants) as a direct consequence of the existence in yeasts of a stable haploid lifestyle. In most multicellular organisms and in diploid yeasts, cells in G_1 have a diploid number of chromosomes (2C or 2n). In haploid yeast, those cells have one of each chromosome (1C or 1n), thus allowing analysis of a given mutation (usually a conditional mutation, e.g., thermosensitive or ts) of a particular gene in the absence of an extra copy of that wild-type gene. Isolation of many of these mutations allowed us to understand how the control of cell cycle progression functions (Hartwell *et al.,* 1970, 1974; Nurse, 1975). These mutations were indeed the basis for the cloning and functional analysis that have provided the frame of our current knowledge about the cell cycle. These major contributions to cell biology started in the early 1970s with the isolation of *cdc* mutants in *S. cerevisiae* (Hartwell *et al.,* 1970), followed by the isolation of *cdc* mutants in *Schizosaccharomyces pombe* (Nurse, 1975), a model yeast organism employed in major contributions to cell cycle control analysis. Based on the understanding that cellular division is essential for proliferation and that it could be compromised in these haploid unicellular microorganisms, conditional mutants were isolated that were able to grow under permissive conditions—i.e., the permissive temperature for ts mutants (usually 25°C)—and that were blocked in cell cycle progression and cellular proliferation under restrictive conditions (usually 37°C for ts mutants). *cdc* mutants show a characteristic phenotype of continued growth in the absence of cell division/cell proliferation.

A central aspect in the cell cycle is the dependence of the different stages (G_1, S phase, G_2, and M phase) that cells pass through once they have been committed to cell division, such that the initiation of late events depends on the completion of early events. This dependence implies a molecular coordination so that cell cycle events do not occur until previous ones have been properly completed. This molecular coordination involves intrinsic controls that regulate transitions from one stage to another. The existence of these intrinsic control mechanisms explains why sister chromatid condensation only occurs when DNA synthesis has been completed and why dividing cells fail to undergo cytokinesis before chromosome segregation has occurred. On the other hand, this dependence is a requirement for cell viability since, for instance, if cytokinesis occurs before chromosome segregation at least one of the daughter cells will be unviable as a consequence of chromosome misegregation. Finally, the interdependence or the existence of these intrinsic control mechanisms in the cycle indicates plasticity. The cell division cycle is not a rigorously established sequence of events but rather a number of very different and mutually interdependent processes with the same purpose: to replicate a mother cell into two daughter with the same replicating capability. What are the mechanisms involved in the control of the cell division cycle? What are the components of these mechanisms? How are they regulated to provide plasticity of the cell cycle?

The most widely accepted and experimentally sound hypothesis explains the cell division cycle control as the result of two interdependent mechanisms—a set of kinases called CDKs that control cell cycle progression, working as an engine, and a set of intrinsic controls called "checkpoint controls" that act as security systems regulating, if required to do so, progression through the cell cycle by modulating CDK activity. These checkpoint controls are redundant in the sense that in rapidly replicating cells there is usually no need for their action.

CDK activity oscillates along the cell cycle. This is the result of a combination of regulatory mechanisms that control their activity. First, CDKs are complexes of a catalytic subunit and a special class of proteins called cyclins (Evans *et al.,* 1983). The catalytic subunit only functions when it is complexed to its cyclin partner. Second, phosphorylation and the dephosphorylation of specific residues in the catalytic subunit regulate CDK activity positively and negatively (Ducommun *et al.,* 1991; Gould *et al.,* 1991; Mendenhall and Hodge, 1998). Both regulatory mechanisms, plus the synthesis and proteolysis of components and postranslational changes, ensure a fine modulation of the dividing activity.

CDKs promote phosphorylation of specific substrates in each phase of the cell cycle, activating or deactivating secondary effectors that will promote or inhibit a given transition event. A special class of CDK, called SPF (S phase promoting factor), promotes the initiation of DNA replication at the G_1 to S phase transition and, once this has been initiated, inhibits the formation of the complex needed for replication before cells exit mitosis. CDKs also promote the disassembly of the nuclear envelope at the G_2 to M phase transition and chromosome condensation.

B. Oscillation of Cyclin-Dependent Kinase Activity

CDKs were identified by genetic analysis in *S. cerevisiae* (Hartwell *et al.,* 1974; Nasmyth and Reed, 1980; Lorincz and Reed, 1984) and in *S. pombe* (Nurse and Thuriaux, 1980; Hindley and Phear, 1984). Coincident with the genetic approach, biochemical research on frog eggs suggested that the cycle was driven by MPF (maturation promoting factor), a cell cycle engine that is composed of two subunits, cyclin B and Cdc2/Cdc28, which are the CDKs of mitosis in fission and budding yeast (Dunphy *et al.,* 1988; Lohka *et al.,* 1988). CDKs have been discovered in all eukaryotes studied to date, suggesting the universality of the cell cycle control mechanism. In *S. cerevisiae* there are six CDKs (*KIN28, PHO85, SSN3, CTK1, CDC7,* and *CDC28*) (Andrews and Measday, 1998), but only *CDC28* plays a general role in controlling progression through the cell cycle (Reed and Wittenberg, 1990; Nasmyth, 1993).

The *CDC28* gene product also known as CDK1, the catalytic subunit of the CDK, is essential in budding yeast. Different *CDC28* conditional mutants arrest their cell cycle in G_1 (prior to START) (Reed and Wittenberg, 1990), or in G_2 (before entry into mitosis) (Piggott *et al.,* 1982; Reed and Wittenberg, 1990; Surana *et al.,* 1991). Several of these mutants also show defects in chromosome stability (Devin *et al.,* 1990), spindle pole body segregation (Lim *et al.,* 1996a), and meiosis (Shuster and Byers, 1989). The Cdc28 protein is present throughout the cell cycle and its activity is controlled at the postranslational level. This kinase has little or no activity as a monomer (Wittenberg and Reed, 1988), and full activation of Cdc28 requires both cyclin binding and phosphorylation and dephosphorylation of key residues (Mendenhall and Hodge, 1998). Nevertheless, a different control level is achieved by a special class of proteins that bind and inhibit CDK activity. These proteins are called CDK kinase inhibitors CKIs).

Cyclins present a common domain known as the "cyclin box" (Morgan, 1995) that is required for binding the catalytic subunit to form the CDK complex. Nine cyclin proteins that bind Cdc28 have been identified in *S. cerevisiae*. They are classified in two different groups according to their function and structure: G_1 cyclins (*CLN1–CLN3*) (Hadwiger *et al.,* 1989b) and B-type cyclins (*CLB1–CLB6*) (Richardson *et al.,* 1992), sharing homology with metazoan B-type cyclins (Richardson *et al.,* 1992). CDK1/G_1 cyclin complexes regulate progression through G_1, whereas CDK1/cyclin B complexes regulate the successive events from late G_1 (the initiation of DNA replication) to the end of mitosis. Thus, it is reasonable to envisage that in budding yeast there are nine different CDKs that result from the combination of one catalytic subunit, Cdc28, and nine cyclins, Cln's and Clb's (Fig. 3). Progression through cellular division implies that overlapping waves of CDKs ensure the phosphorylation of different substrates at each transition point in the cell cycle. These waves increase the levels of kinase activity from late G_1 to mitotic exit (Nasmyth, 1996). Thus, the cell division cycle can be understood as a CDK cycle (Nasmyth, 1996; Stern and Nurse, 1996).

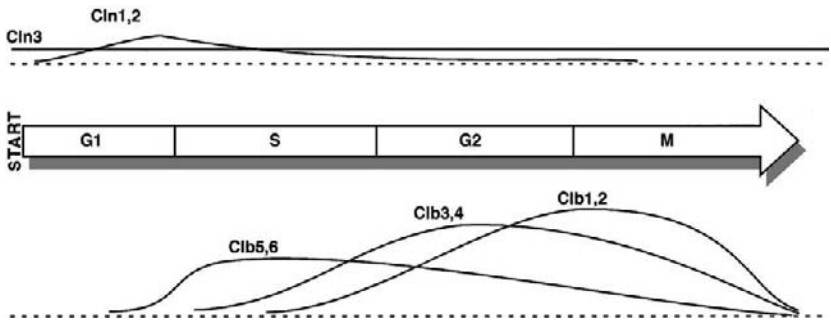


FIG. 3 Waves of different CDKs throughout the cell division cycle of *S. cerevisiae*. CDK1 complexed with different cyclin subunits conform fully active kinases whose activities overlap through different stages of the cell cycle.

Each cyclin confers a limited period of activity and specificity to the CDK1 kinase (Lew and Reed, 1992; Arellano and Moreno, 1997). The Cln cyclin family is essential to *S. cerevisiae*. Triple *cln* mutants arrest in G_1 before START (Richardson *et al.,* 1989), although loss of any two *CLN* genes is tolerated. This means that any of the G_1 cyclins can promote passage through G_1 (Hadwiger *et al.,* 1989b; Richardson *et al.,* 1989; Cross, 1990). In contrast to *CLN1* and *CLN2,* both messenger and protein levels of *CLN3* are expressed at constant levels along the cell cycle (Wittenberg *et al.,* 1990; Tyers *et al.,* 1992; Cross and Blake, 1993). Genetic and biochemical approaches indicate that the CDK1/Cln3 complex has a more relevant role than CDK1/Cln1 or Cln2 complexes, acting as an activator of Cln1,2 complexes and through its action on Swi4–Swi6 binding factor (SBF) (Cross, 1988; Nash *et al.,* 1988; Tyers *et al.,* 1993; Dirick *et al.,* 1995; Levine *et al.,* 1996).

CLN1 and *CLN2* are "true" cyclins because they are transcribed periodically during the cell cycle. The expression of both genes peaks in G_1 (Wittenberg *et al.,* 1990), and MCB (MluI-cell cycle box) and SCB (Swi4–Swi6 cell Cycle box) promoter elements are required for proper expression (Cross *et al.,* 1994; Stuart and Wittenberg, 1994). Other CDK1/cyclin complexes control the timing of *CLN1* and *CLN2* expression: whereas CDK1/Cln3 turns transcription on, CDK1/Clb1–4 turn transcription off (Amon *et al.,* 1994). Cln2 protein levels correlate with mRNA levels (Wittenberg *et al.,* 1990), mostly because Cln2 protein instability is regulated or promoted by CDK1/cyclin complexes (Lanker *et al.,* 1996). Phosphorylated Cln2 protein is targeted for degradation through the proteasome in a SCF-dependent manner (Skp1-Cullin-Fbox protein) (Lanker *et al.,* 1996; Deshaies, 1999). Genetic and biochemical evidence suggests an important role for timely and accurate passage through START for CDK1/Cln1 and Cln2 complexes (Hadwiger *et al.,* 1989b; Dirick *et al.,* 1995; Stuart and Wittenberg, 1995). *Saccharomyces cerevisiae* cells require passage through START in order to

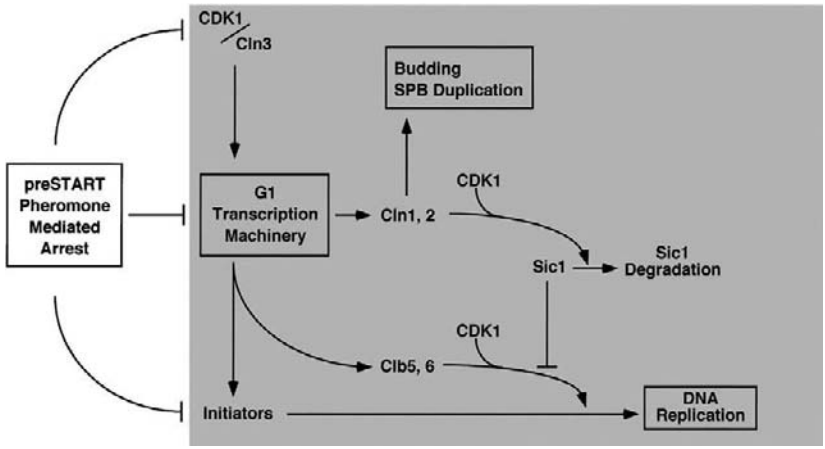


FIG. 4 Events involved in G_1 in repressing or inducing reentry of the cell cycle from a pheromone-induced arrest in *S. cerevisiae* cells.

proceed with bud formation, spindle pole body (SPB) duplication, and initiation of DNA replication. At least one CDK1/Cln kinase activity is required prior to the initiation of DNA replication, SPB duplication, and bud emergence (Fig. 4) (Benton *et al.,* 1993; Cvrckova and Nasmyth, 1993). Of particular interest is the role of CDK1/Cln complexes in DNA replication, which seems to be indirect by triggering the proteolysis of the CDK1/Clb inhibitor Sic1 (Schwob *et al.,* 1994; Verma *et al.,* 1997). In addition, CDK1/Cln1 and Cln2 activity is required for repression of the pheromone signaling pathway at START (Oehlen and Cross, 1994).

Once the Sic1 CDK kinase inhibitor has been destroyed by the proteasome, CDK1/Clb kinases initiate their pattern of sequential activities (Fig. 5). From a functional standpoint, CDK1/Clb kinases can be subdivided into three groups: Clb5–6, Clb3–4, and Clb1–2. Clb5 and Clb6 are similar at the primary sequence level (Costanzo *et al.,* 2000). *CLB5* and *CLB6* transcriptional patterns of expression are also very similar, peaking at the G_1 to S phase transition (Epstein and Cross, 1992; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993). Expression of both cyclin genes is probably regulated by the MCB sequence present in their promoters, and their transcription is positively regulated by CDK1/Cln3 (Dirick *et al.,* 1995; Stuart and Wittenberg, 1995). The primary role of CDK1/Clb5 and Clb6 complexes is to trigger the initiation of DNA replication (Oehlen *et al.,* 1998), probably by acting directly on the prereplicative complexes at replication origins (see Section IV.B.2). However, the details of the mechanism of CDK1/Clb5–6 action remain to be elucidated. In addition to this role, both CDKs have been implicated in the mechanism of prevention of rereplication within the same cell cycle. CDK1/Clb5–6 complexes have a negative effect on CDK1/Cln2 complex

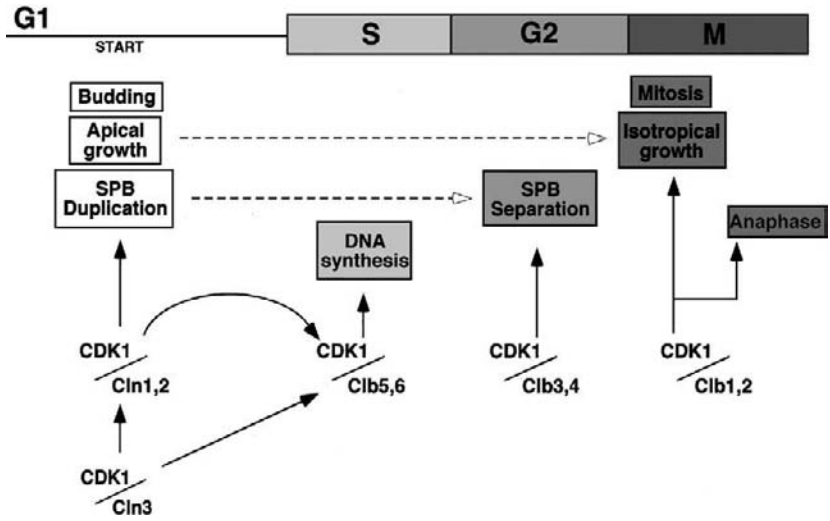


FIG. 5 Waves of different cyclin-dependent kinases ensure the appropriate order of events along the cell division cycle progression in the budding yeast *S. cerevisiae*.

formation (Basco *et al.,* 1995) and a positive role in spindle assembly and proper orientation (Schwob and Nasmyth, 1993; Segal *et al.,* 2000).

CLB3 and *CLB4* transcripts appear at the beginning of S phase and remain at high levels until late anaphase (Surana *et al.,* 1991; Epstein and Cross, 1992; Fitch *et al.,* 1992). These patterns of expression correlate with CDK1/Clb3- and Clb4-associated kinase activity (Grandin and Reed, 1993). The mechanism(s) responsible for turning these genes on and off remains unknown. The periodicity of Clb3 and Clb4 proteins is at least in part due to their rapid degradation, and the presence of a consensus "destruction box" motif at the amino terminus of both polypeptides may be responsible (Lew and Reed, 1992). Given their timing of appearance, it has been suggested that CDK1/Clb3 and Clb4 may have a role in S phase, but in any case they are preferentially required for spindle assembly (Segal *et al.,* 2000).

The last pair of cyclin genes, *CLB1* and *CLB2,* are mainly expressed in G_2 and mitosis, peaking before anaphase (Ghiara *et al.,* 1991; Surana *et al.,* 1991; Fitch *et al.,* 1992; Richardson *et al.,* 1992). Their expression is probably regulated by Mcm1 (Oehlen *et al.,* 1998). CDK1/Clb2 is the predominant kinase in mitotic-arrested cells compared with CDK1/Clb1-associated activity (Grandin and Reed, 1993). In contrast, the latter is the predominant meiotic complex (Grandin and Reed, 1993; Cooper *et al.,* 2000). Genetic analysis has shown that *CLB2* has a more relevant role than any other single cyclin gene in *S. cerevisiae. CLB2* deletion mutants are larger than wild-type cells and show defects in progressing into mitosis (Surana *et al.,* 1991; Richardson *et al.,* 1992). In contrast, *CLB1* mutants have no

obvious mitotic phenotype but, consistent with a role in the meiotic cycle for this cyclin, the normal execution of meiosis I and II requires CDK1/Clb1 and Clb4 kinases (Shuster and Byers, 1989; Grandin and Reed, 1993; Dahmann and Futcher, 1995). These observations support the idea that the CDK1/Clb2 complex is the major mitosis promoter factor and that the CDK1/Clb1 complex plays a minor role in entry into mitosis. Consistent with this notion, CDK1/Clb2 plays an important role in spindle elongation and promotes isotropic growth of buds (Lew and Reed, 1993). This kinase is also involved in the negative regulation of SBF-promoted transcription (Amon *et al.,* 1993) and bud emergence (Ghiara *et al.,* 1991; Booher *et al.,* 1993; Surana *et al.,* 1993).

CDK1/Clb2 inactivation is essential for exit from mitosis (King *et al.,* 1996) and therefore for establishing a G_1 period in the cell division cycle (see Section II.C). The amino acid sequence of Clb2 contains a destruction box involved in the disappearance of Clb2 protein at the end of mitosis (Amon *et al.,* 1994; Baumer *et al.,* 2000). Clb2 protein degradation is mediated through polyubiquitination by anaphase promoting complex and proteolysis by the proteasome (Zachariae and Nasmyth, 1999; Tyers and Jorgensen, 2000). The associated kinase activity of CDK1/cyclin complexes can be regulated posttranslationally by phosphorylation of the Cdc28-catalytic subunit and/or by association with other proteins, both acting as positive or negative effectors (Fig. 6).

Cdc28 phosphorylation has been documented at two different amino acid sites: threonine-169 (T^{169}) and tyrosine-19 (Y^{19}). The T^{169} residue must be phosphorylated for the CDK-associated kinase to be fully active. Consistent with this, substitution of the T^{169} by an alanine (nonphosphorylatable residue) generates a protein that can neither support cell proliferation (Cismowski *et al.,* 1995; Lim *et al.,* 1996b) nor be activated *in vitro* (Deshaies and Kirschner, 1995). Cdc28 T^{169} phosphorylation is catalyzed by the Cak1 kinase (synonymous with *CIV1*), both in its monomeric Cdc28 form and in CDK1/cyclin complexes (Espinoza *et al.,* 1996; Kaldis *et al.,* 1996; Thuret *et al.,* 1996), although only CDK1/cyclin complexes are activated upon phosphorylation by Cak1 (Espinoza *et al.,* 1996; Kaldis *et al.,* 1996; Thuret *et al.,* 1996). There is no evidence suggesting a role in cell cycle regulation for T^{169} phosphorylation, but CDK1/cyclin complexes formed with Cln cyclins are active even in the absence of such phosphorylation, whereas complexes formed with Clb cyclins require T^{169} phosphorylation to become fully active (Cross and Levine, 1998).

Y^{19} phosphorylation is cell cycle regulated. Y^{19} is phosphorylated at maximum levels during S phase and G_2 intervals, but most CDK molecules remain dephosphorylated at this tyrosine residue during mitosis and G_1 (Amon *et al.,* 1992; Sorger and Murray, 1992). Tyrosine phosphorylation explains how mitotic CDK is regulated in multicellular systems and in fission yeast. In *S. pombe,* when the equivalent tyrosine (Y^{15}) is phosphorylated, CDK kinase remains inactive and entry into mitosis is delayed. Only when the residue is dephosphorylated does

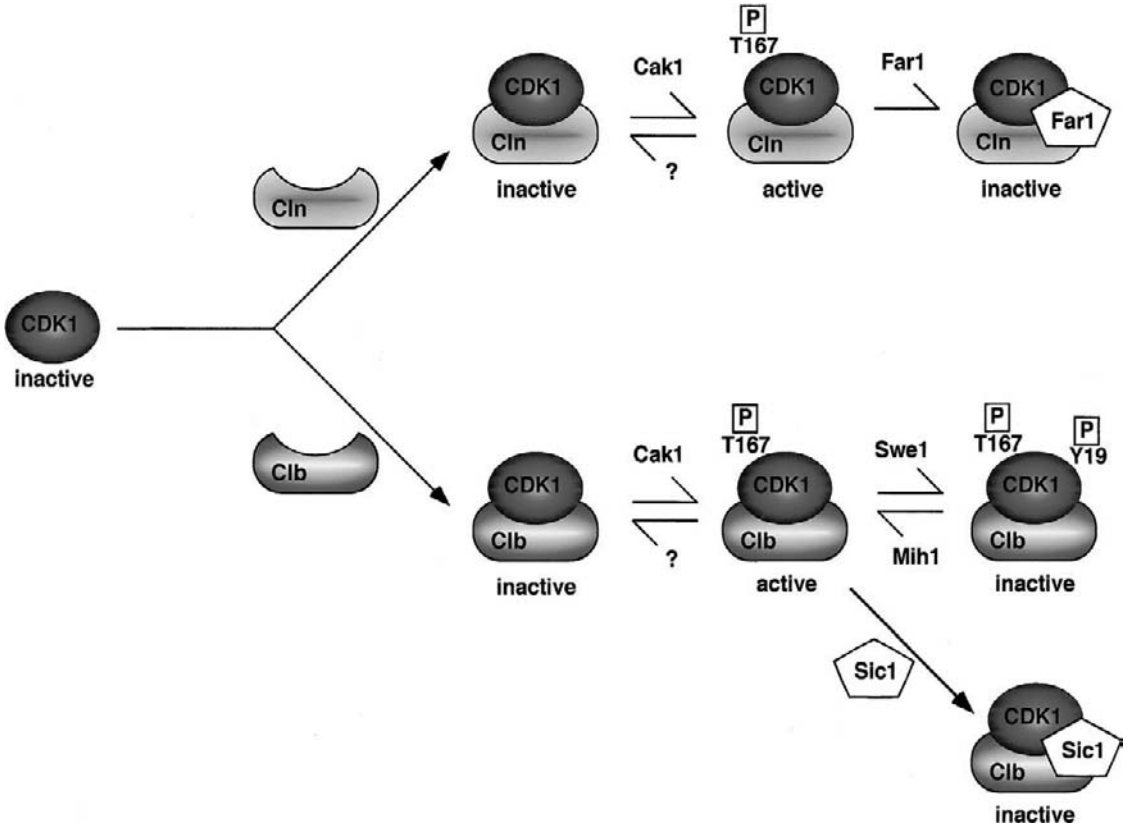


FIG. 6 Regulation of CDK1/Cln and CDK1/Clb activities. The activity of CDKs depends on positive and negative signals. Phosphorylation of T167 by the Cak1 kinase is required for CDK1 activation. On the other hand, Swe1 phosphorylation downregulates CDK1 activity. Specific inhibitors interact with CDK1/Cln (Far1) and CDK1/Clb (Sic1).

the complex become active and the cells undergo mitosis. This mechanism is controlled by the action of the Wee1 kinases and the Cdc25 phosphatases (Lew and Kornbluth, 1996; MacNeill and Nurse, 1997). Nevertheless, in *S. cerevisiae* this control mechanism appears to be redundant since a Cdc28 Y19F mutation that mimics the unphosphorylated form does not affect any aspect of the cell division cycle analyzed to date (Amon *et al.,* 1992; Sorger and Murray, 1992). Despite this difference, genes homologous to the Wee1 kinase (*SWE1*) and to the Cdc25 phosphatase (*MIH1*) have been identified in this budding yeast (Russell *et al.,* 1989; Booher *et al.,* 1993). Two studies have related the Swe1 kinase to the *in vivo* inhibition of CDK1/Clb1–4 complexes (Booher *et al.,* 1993; Lim *et al.,* 1996a). The same enzyme is unable to phosphorylate CDK1/Cln2 complexes *in vitro* (Booher *et al.,* 1993).

CDK1/cyclin complex activity can also be inhibited through the action of CKI proteins (Peter and Herskowitz, 1994b). Three CKIs have been identified in *S. cerevisiae:* Far1 (Tyers and Futcher, 1993; Peter and Herskowitz, 1994a), Sic1 (Mendenhall, 1993; Schwob *et al.,* 1994), and Cdc6 (Calzada *et al.,* 2001). Far1 and Sic1 have no homology to each other or to CKI proteins identified in mammals. However, the Sic1 inhibitor shows limited homology to the Rum1 protein *S. pombe,* a central CKI regulator of the G_1 phase in the fission yeast (Sánchez-Díaz *et al.,* 1998). In *S. cerevisiae,* Far1 specifically inhibits CDK1/Cln complexes, whereas Sic1 inhibits Clb-associated CDK complexes (Peter and Herskowitz, 1994b; Schwob *et al.,* 1994). Cdc6 participates in the inhibitory pathway leading to CDK inactivation at the exit from mitosis, acting in parallel to Sic1 in a less efficient manner (Calzada *et al.,* 2001). Remarkably, Cdc6 is a member of a well-conserved family of proteins involved in the initiation of DNA replication (see Section III.B) (Dutta and Bell, 1997). Far1, Sic1, and Cdc6 protein levels oscillate along the cell cycle. Maximum levels are achieved from late mitosis to the G_1 to S phase transition (McKinney *et al.,* 1993; Donovan *et al.,* 1994; Schwob *et al.,* 1994; Piatti *et al.,* 1995). In addition to these regulators, it has been described that *CKS1* encodes an essential protein with a role in regulating the proper assembly of CDK1/cyclin complexes. Cks1 is the budding yeast homolog of the *S. pombe* Suc1 protein (Brizuela *et al.,* 1987; Hindley *et al.,* 1987) and is essential for Cdc28 function (Hadwiger *et al.,* 1989a), most likely acting as an assembly factor for CDK1/cyclin complexes.

C. Importance of G_1 in the Initiation of DNA Replication

Cell division events are restricted by the once per cell cycle rule. Once a given transition in cell cycle progression has been completed, events prior to that particular transition do not occur again until nuclear division has been successfully finished and cell division starts a new cycle. Single DNA replication is required in order to ensure a stable propagation of the genome to daughter cells. How is genome

duplication restricted to a single replication every cell cycle? Given the peculiarity of the eukaryotic genome, composed of several chromosomes and a high number of replication origins, which ensures that the whole genome is replicated, a tight DNA control of replication is imposed on each replication origin. Once DNA synthesis has been triggered from a given origin, reinitiation is actively prevented until the cell exits mitosis. By restricting origin of replication firing to only once within the S phase, cells ensure that a only one round of S phase occurs per cell cycle. How is the initiation of DNA synthesis from origins controlled with such accuracy? Recent advances in our understanding of the mechanism of initiation of DNA replication suggest that CDK-associated kinase activity participates actively in preventing rereplication. Thus, CDK1/Clb complexes play a dual role in DNA replication: First, they are required for the activation of competent origins of replication and then, once such origins have been fired, they prevent the formation of competent origins until the cells have segregated the replicated genome during mitosis.

The positive role of CDK1/cyclin complexes in the initiation of DNA replication is inferred from genetic approaches. Analysis of conditional *cdc28* mutations indicates that an active kinase is required for the initiation of S phase (Hartwell *et al.,* 1974). The cyclin partners for the activation of DNA replication are Clb5 and Clb6, but any other Clb protein in the absence of Clb5 and Clb6 is able to promote DNA replication (Epstein and Cross, 1992; Richardson *et al.,* 1992; Schwob and Nasmyth, 1993; Schwob *et al.,* 1994). CDK1/Clb5 and Clb6 are activated first in late G_1 to promote S phase by triggering initiation (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). CDK1/Clb1–4 are activated later in S and G_2 to promote later cell cycle events, including entry into mitosis (Surana *et al.,* 1991; Richardson *et al.,* 1992). The distinction between them in promoting the initiation of DNA replication might be due to their order of appearance rather than their substrate specificity since in the absence of both Clb5 and Clb6, one of the others can act redundantly (Schwob and Nasmyth, 1993; Amon *et al.,* 1994; Schwob *et al.,* 1994). Hence, CDK1/Clb complexes able to promote S phase are continuously present from late G_1 until the end of mitosis, when CDK1/Clbs are inactivated (Nasmyth, 1996).

Despite the continuous presence of CDKs able to promote DNA replication, a new round of genome duplication cannot take place until cells progress through the G_1 phase of the next cell cycle. This is due to the presence of the same promoting activity, because CDK1/Clb1–6 kinases are required to prevent reinitiation of DNA replication within the same cell cycle (Dahmann *et al.,* 1995). All experimental evidence suggests that this is a general mechanism for eukaryotes, conserved from yeast to humans (Broek *et al.,* 1991; Hayles *et al.,* 1994; Dahmann *et al.,* 1995; Singer *et al.,* 1996; Itzhaki *et al.,* 1997).

This model (Fig. 7) is based on the alternance of an interval without CDK1/cyclin activity (CDK off)—G_1—with intervals with CDK1/cyclin activity (CDK on), including S, G_2, and M phases. There are two strong lines of evidence supporting

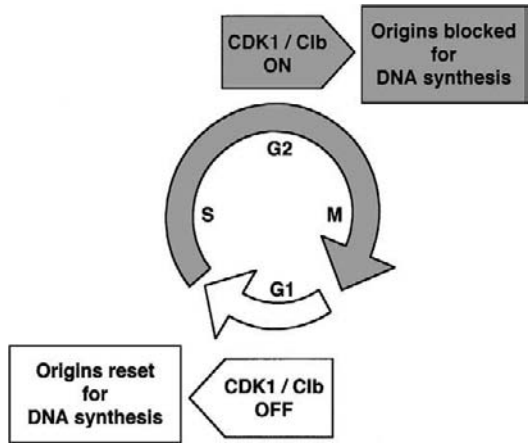


FIG. 7 Alternance of DNA replication and mitosis is ensured by cycles of periodic activation and inactivation of CDK1 in *S. cerevisiae*. The absence of CDK1 activity is required to form pre-RC complexes at origins and the presence of CDK1 activity is required for cells to progress to S phase.

this model. First, ectopic activation of CDK1/Clb2 in G_1 prevents DNA replication (Detweiler and Li, 1998). Second, a transient inhibition of the CDK1/Clb kinase in cells blocked in mitosis leads to an extra round of DNA replication (Dahmann *et al.,* 1995). These data are consistent with the importance of a G_1 period lacking CDK promoting activity to allow the genome to be competent for replication (see Sections IV.A and IV.B) and with the idea that reinitiation is directly blocked during S, G_2, and M phase by the same CDK complexes.

D. Regulation of Genes Involved in the Initiation of DNA Replication

Initiation of DNA replication requires the formation at each origin of replication of a protein complex formed by many different gene-encoded products. The expression of these genes is cell cycle regulated and periodically limited (for some of them) to telophase at the end of mitosis or (for the rest) to the G_1 phase. The previously mentioned protein complex is called the prereplicative complex (pre-RC) and establishes origin of replication competence to be activated by S-CDK. Two different conditions are required for cells to initiate DNA replication: pre-RCs formed at origins (see Section III.B) and S-CDK-mediated activation of DNA synthesis at origins (see Section III.C).

Cyclin cells overlap consecutive cell division cycles in such a way that regulation of the G_1 stage starts at the end of the previous mitosis. In this context it should be recalled that some genes participating in DNA replication initiation are transcribed

TABLE I
Transcription Factors Regulating the Expression of Genes Involved in the Initiation of S Phase

Transcription factor	Genes		
ECB (Mcm1)	*ACE2*	*MCM7*	
	SWI5	*MCM5*	
	SWI4	*CDC6*	
	CLN3		
Swi5/Ace2	*SIC1*		
	CDC6		
SBF (Swi6/Swi4)	*CLN1*		
	CLN2		
MBF (Swi6/Mbp1)	*CLB5*	*DBF4*	*POL1*
	CLB6	*MCM5*	*POL2*
	CDC6	*MCM7*	*RFA1*
	ABF1	*PRI1*	*RFA2*
	ORC6	*PRI2*	*RFA3*
	MCM10		

during telophase (late in mitosis) or even earlier (Table I). This is the case for *CDC6, CDC46* (*MCM5*), *CDC47* (*MCM7*), and the transcription factors encoded by *SWI4* and *SWI5* regulating the expression of other genes involved in DNA replication. The transcription of *SWI5* is regulated by Mcm1, a transcriptional activator of important genes at the G_2/M transition such as *ACE2* (Fitch *et al.,* 1992) or *CLB1* and *CLB2* (Ghiara *et al.,* 1991; Fitch *et al.,* 1992; Richardson *et al.,* 1992; Wynne and Treisman, 1992; Surana *et al.,* 1993). Mcm1 also regulates the expression of *CDC6, CDC46/MCM5, CDC47/MCM7, CLN3,* and *SWI4* at the M to G_1 transition point (Mendenhall and Hodge, 1998). In these cases, Mcm1- regulated expression depends on the presence of an early cell cycle box sequence (McInerny *et al.,* 1997).

The expression of some of these genes might be even more complex, involving several transcriptional activators and/or inhibitors. For example, transcription of *CDC6* at the M/G_1 transition is regulated by Mcm1 but also depends on Swi5 (Zwerschke *et al.,* 1994; Piatti *et al.,* 1995; Aerne *et al.,* 1998), once this transcription factor has been activated at the end of mitosis (Nasmyth *et al.,* 1990). Remarkably, *SICI* M/G_1 transcription is also dependent on Swi5 and Ace2 (Toyn *et al.,* 1995; Knapp *et al.,* 1996). Finally, in cells reentering the cell cycle, *CDC6, CDC46, CDC47,* and most of the genes involved in the initiation of DNA replication show a second transcription peak late in G_1 (Spellman *et al.,* 1998), most likely regulated by MCB sequences present in their promoters (Zhou and Jong, 1990; Bueno and Russell, 1992; Koch *et al.,* 1993; Piatti *et al.,* 1995; Toone *et al.,* 1997; Mendenhall and Hodge, 1998). As mentioned previously, the activation of CDK1/Cdc28 at START is essential for the initiation of genome replication (see

Section II.B). CDK1/Cln3-dependent G_1 phase transcription (Tyers *et al.,* 1993; Levine *et al.,* 1996) is responsible for the expression of the Cdc28 cyclin partners *CLN1* and *CLN2* (Wittenberg *et al.,* 1990) and *CLB5* and *CLB6* (Kuhne and Linder, 1993; Schwob and Nasmyth, 1993), as well as for the expression of genes directly involved in the formation of pre-RCs necessary to initiate DNA replication (Tyers *et al.,* 1993; Levine *et al.,* 1996; McInerny *et al.,* 1997).

CDK1/Cln3-dependent G_1 phase transcription is generally attributed to the regulatory action of two related transcription factors—SBF and MBF—that are responsible for most of the periodic late G_1 mRNA production. SBF is the most important transcription complex controlling the expression of *CLN1* and *CLN2* (Nasmyth and Dirick, 1991; Ogas *et al.,* 1991). SBF recognizes a specific DNA sequence called SCB. SBF is a complex formed by a regulatory protein, Swi6, and a sequence-specific DNA binding protein, Swi4 (Andrews and Moore, 1992; Primig *et al.,* 1992; Sidorova and Breeden, 1993). MBF is also a transcription complex formed by the regulatory protein Swi6 and a Swi4-related DNA binding protein called Mbp1. MBF binds a DNA sequence element called MCB that can be found in the promoter sequences of most DNA replication genes (Koch *et al.,* 1993; Toone *et al.,* 1997), including *CLB5* and *CLB6,* whose expression is thought to be regulated by this complex (Toone *et al.,* 1997). It is believed that CDK1/Cln3-mediated phosphorylation of SBF and MBF directly activates these complexes once cells have passed START, although direct evidence is lacking (Mendenhall and Hodge, 1998).

A summary of the regulatory events regarding transcriptional regulation related to S phase initiation is provided in Fig. 8 and Table I.

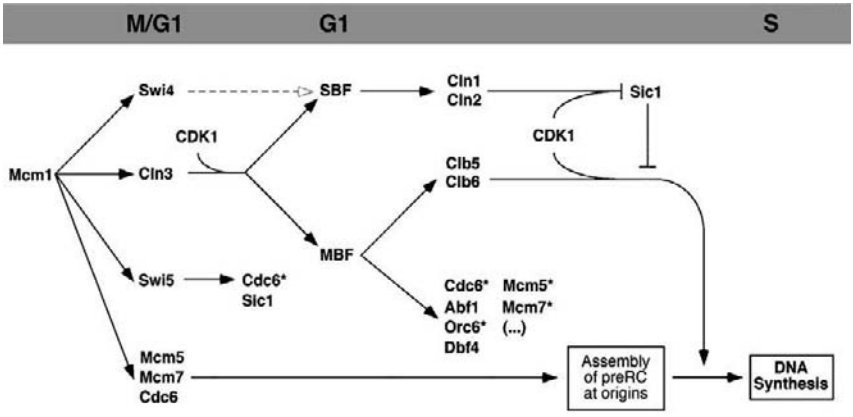


FIG. 8 Transcriptional regulation of genes involved in the initiation of DNA replication. Asterisks indicate those genes that although transcribed in M/G_1 in logarithmically growing cells are also transcribed in G_1 in the case of reentering the cell cycle from a G_1 arrest.

III. Elements Required for Proper Initiation of DNA Replication

Initiation of DNA replication in eukaryotes is possible due to the sequential cooperation of three different elements—a *cis*-acting element, from which DNA synthesis is initiated, known as the origin of replication or replicator; a *trans*-acting protein complex known as the initiator or pre-RC, which is assumed to be a landing path for the replication machinery (see Section IV.C); and S-CDK, the regulatory activator of the initiation event. Thus, for a comprehensive understanding of the molecular events involved in the initiation of genome replication in eukaryotes it is necessary to study how eukaryotic origins of replication are organized. Given the focus of this review, we concentrate on the replication origin properties in *S. cerevisiae*.

A. Origins of DNA Replication

Initiation of genome replication is directed by specific DNA sequences, origins of replication or replicators. The site of DNA unwinding and initial DNA synthesis is localized within or near these replicators (Bielinsky and Gerbi, 1999). In contrast to the well-characterized replicators of bacterial chromosomes, phages, and eukaryotic viruses, little is known about the structure and function of eukaryotic chromosomal replicators. Among the latter, the best characterized are those of the budding yeast *S. cerevisiae*. In the following sections we summarize current knowledge about chromosomal replicators in yeast and multicellular eukaryotes.

1. Structure and Function of *S. cerevisiae* Autonomously replicating sequence Elements

Budding yeast origins of replication were initially identified as DNA sequences that allowed the maintenance of plasmids in yeast. These DNA fragments were able to direct plasmid replication and thus were called autonomously replicating sequences (ARSs) (Kingsman *et al.,* 1979; Stinchcomb *et al.,* 1979; Struhl *et al.,* 1979; Beach *et al.,* 1980; Chan and Tye, 1980; Tschumper and Carbon, 1980). Later, two-dimensional gel analysis showed that most ARSs effectively drove the initiation of DNA replication at chromosomal sites and that ARSs and origins of replication colocalize at chromosomal locations (Brewer and Fangman, 1987; Huberman *et al.,* 1987).

Analysis of ARS elements has revealed the core sequences required for an origin of replication to be functional (Fig. 9) (Newlon and Theis, 1993). Sequence comparison of several ARSs reveals that they are more A/T rich than the surrounding DNA and, perhaps more significant, that they share a sequence of 11 base pairs (bp) known as ACS (ARS consensus sequence) (Newlon and Theis, 1993).

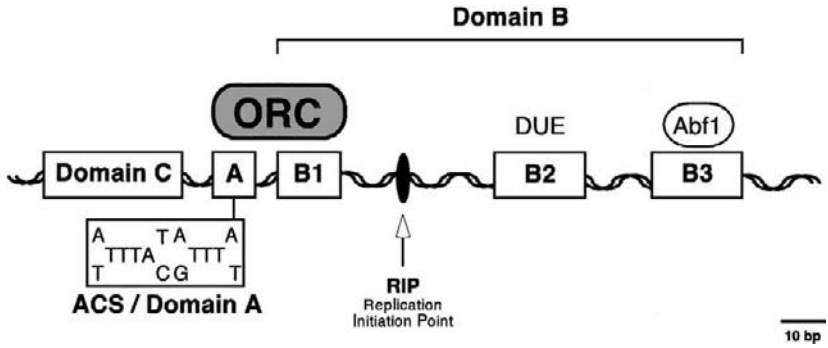


FIG. 9 The structure of the *S. cerevisiae ARS1* replication origin.

Deletion and mutational analysis of these sequences revealed that this 11-bp element is essential for the replication function (Van Houten and Newlon, 1990; Marahrens and Stillman, 1992; Deshpande and Newlon, 1992; Huang and Kowalski, 1993). ACS elements are also known as domain A of origins of replication (Fig. 9). This domain A is necessary, but not sufficient, for replicator function since both 3′ and 5′ flanking sequences contribute to ARS functionality (Stillman, 1996). These upstream and downstream elements are usually 50–100 bp adjacent to the ACS. Sequences 3′ to the T-rich strand of the ACS are known as domain B, and sequences 5′ to the T-rich strand are called domain C (Celniker *et al.,* 1984; Rowley *et al.,* 1994). Detailed studies performed on some ARSs suggest that domain B can be subdivided into different but not essential subdomains (B1–B3 in Fig. 9) (Marahrens and Stillman, 1992; Rao *et al.,* 1994; Theis and Newlon, 1994). Each of these subdomains appears to stimulate the replicative function, but their relative contribution to origin efficiency changes from one ARS to another (Marahrens and Stillman, 1994; Rao *et al.,* 1994). These subdomains can be grouped in sequence-specific elements, acting as protein-binding sequences, and non-sequence-specific elements, which are thought to act as DNA unwinding sites (Theis and Newlon, 1994). A leading strand site was recently identified at ARS1, located close to a potential DNA-unwinding element—the B2 element of ARS1 (Bielinsky and Gerbi, 1999).

Origins of replication, or ARSs, are the landing paths for many proteins involved in DNA synthesis initiation. The ACS element is bound by a multiprotein complex known as the origin recognition complex (ORC) (see Section III.B) (Bell and Stillman, 1992), and the B3 domain is where the transcription factor Abf1 binds (Abf1 stands for ARS1 binding factor 1) (Shore and Nasmyth, 1987; Rowley *et al.,* 1994; Diffley, 1998), although the contribution of this transcription factor to the initiation of replication remains obscure. Nevertheless, a close relationship between transcription and replication has been suggested for several eukaryotic systems (Kitsberg *et al.,* 1993; Giacca *et al.,* 1994; Delgado *et al.,* 1998; Pierron

et al., 1999; Sasaki *et al.,* 1999). Other protein complexes specifically bind different domains in ARSs. These complexes are described in Section III.B.

2. Replication Origins of *S. pombe*

In the fission yeast *S. pombe,* DNA replication seems to be initiated at discrete points, similarly to budding yeast (Caddle and Calos, 1994; Dubey *et al.,* 1994; Okuno *et al.,* 1997). Despite this similarity, research has revealed that the organization of the *cis*-acting elements forming a replicator in *S. pombe* shows significant differences from the *S. cerevisiae* origins of replication. Hence, shorter fission yeast DNA elements able to sustain ARS activity range from 500 to 1500 bp (Maundrell *et al.,* 1988; Dubey *et al.,* 1994; Wohlgemuth *et al.,* 1994; Clyne and Kelly, 1995; Dubey *et al.,* 1996; Kim and Huberman, 1998), in contrast to the characteristic 150-bp length of budding yeast (Newlon and Theis, 1993). However, these fission yeast DNA fragments span a rich A/T region located at intergenic sequences, as in budding yeast (Stillman, 1996). A more significant difference between replicators in these two yeasts is that, in contrast to *S. cerevisiae,* apparently there are no conserved elements in the sequences required for the initiation of replication in *S. pombe* ARSs (Maundrell *et al.,* 1988; Clyne and Kelly, 1995; Dubey *et al.,* 1996; Kim and Huberman, 1998; Gómez and Antequera, 1999). Finally, a recent analysis of the genomic *ars1* replication origin of *S. pombe* indicated that transcription and replication regulatory elements are a few nucleotides away from each other, suggesting a relationship between both processes *in vivo* (Gómez and Antequera, 1999).

3. Replication Origins in Metazoans

The characterization of replication origins in metazoans has been far more elusive than in budding yeasts. It has been difficult to identify and isolate specific replication origins and link them to particular DNA sequences. A significant number of ARSs from *S. cerevisiae* were isolated owing to their capacity to confer autonomous replication on plasmids. A similar strategy has afforded only partial success in mammalian systems (Heinzel *et al.,* 1991). By analysis of DNA fragments that replicate early during the S phase, the first mammalian ORI was isolated from Chinese hamster ovary cells (CHOs) (Heintz and Hamlin, 1982), and this has been subjected to different detailed analyses (Antequera and Bird, 1999). The characterization of CpG islands in mammalian chromosomes led to the discovery that they function as replication origins, and that the islands constitute a significant fraction of all genomic ORIs in CHO and human cell lines (Delgado *et al.,* 1998; Antequera and Bird, 1999). CpG islands contain the promoter of approximately 50% of all mammalian genes (Larsen *et al.,* 1992; Antequera and Bird, 1993), suggesting a functional correlation between transcription and replication, as in fission yeast. Study of the different mammalian ORIs isolated by these three strategies supports

the notion that one of the fundamental differences between metazoans and *S. cerevisiae* lies in the nature of their replicators. In contrast to budding yeast, there are no specific initiation sequences in mammalian ORIs. Furthermore, it has been suggested that origin usage might be determined epigenetically by higher order chromatin structures and perhaps by interaction with the nuclear matrix (Gilbert, 1998).

B. DNA Replication Initiators

Cell fusion experiments initiated by Guttes and Guttes (1969) led to the conclusion that chromatin exists in two different states regarding S phase initiation—competent for replication and incompetent for replication—and that it is at the time of mitosis exit when chromatin transits from the latter state to the former one (Rao and Johnson, 1970). The involvement of an initiator in these cellular events was revealed years later by studies in both *S. cerevisiae* and *Xenopus*. In particular, in budding yeast the existence of a nonnucleosome chromatin-bound structure in origins of DNA replication was observed (Thomas *et al.,* 1984)—a structure that changes during the cell cycle (Brown *et al.,* 1991). Biochemical studies disclosed that the initiator complex binds to the A and B domains of ARSs at their chromosomal locations (Bell and Stillman, 1992; Rao and Stillman, 1995; Rowley *et al.,* 1995). These complexes were called ORC. Genomic footprinting of ARSs revealed a pattern closely related to the footprint obtained with ORC. Furthermore, ORC footprinting is maintained at different stages of cellular division, suggesting that ORC is bound to replicators throughout the cell cycle (Bell and Stillman, 1992; Diffley and Cocker, 1992). Nevertheless, although ORC is maintained, the footprinting at ARSs does change along the cell cycle and is extended in the interval between the end of mitosis and the G_1 to S phase transition (Diffley and Cocker, 1992). Based on these studies, it has been suggested that chromatin has two different states: One defined by the smaller footprint and characterized by a postreplicative complex (post-RC) of proteins bound to origins of replication, and a second one characterized by an extended footprint that is the consequence of the presence of additional components in the protein complex to form the pre-RC. Post-RCs are characteristic of the noncompetent replication state of chromatin, covering the S, G_2, and M phases of the cell cycle, and pre-RCs correspond to the competent replication state of G_1 chromosomes (Diffley *et al.,* 1994) (Fig. 10). Given that transition from the prereplicative to the postreplicative state of chromatin coincides with the G_1/S transition (Santocanale and Diffley, 1996), it was suggested that pre-RCs are substrates for the *in situ* activators driving the initiation of DNA synthesis and that the transition from one complex (pre-RC) to another (post-RC) is a consequence of the initiation reaction (Diffley *et al.,* 1994; Santocanale and Diffley, 1996; Dutta and Bell, 1997). In the following sections, we summarize current knowledge of each of these complexes, their biochemical properties, and their roles in the initiation of DNA replication.

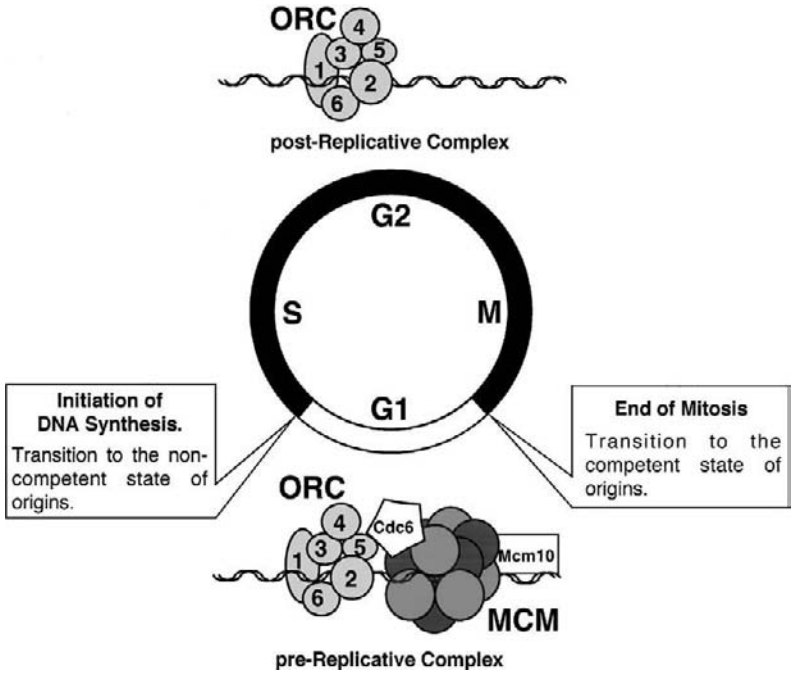


FIG. 10 The DNA replication cycle in *S. cerevisiae* cells. The competence of origins of replication to initiate DNA synthesis is established by the assembly of prereplicative complexes (pre-RCs) of proteins at origins. The initiation of DNA synthesis involves the disassembly of pre-RCs to the postreplicative complexes, returning origins to their inactive state. The exit from mitosis closes the cycle by driving the origins to a new prereplicative state. The initiators conforming each complex are shown.

1. Components of the Postreplicative Complexes at Origins

To date, different proteins have been found to interact with ARSs, but only ORCs have been found bound to ARSs throughout the cell division cycle. The ORC shows the required properties to be considered an eukaryotic initiation complex. First, it recognizes the ACS and the adjacent B1 element of replicators *in vitro* (Bell and Stillman, 1992; Rao and Stillman, 1995; Rowley *et al.,* 1995) and *in vivo* (Liang and Stillman, 1997). These interactions require the presence of intact binding sites on the ACS at ARSs (Rowley *et al.,* 1995; Aparicio *et al.,* 1997; Tanaka *et al.,* 1997). Second, the genes encoding each of the six ORC subunits are essential for cell viability, and the characterization of ts mutants of each of them leads to the conclusion that they are required for efficient initiation of DNA replication at the beginning of S phase (Li and Herskowitz, 1993; Loo *et al.,* 1995; Hori *et al.,* 1996; Klemm *et al.,* 1997). Consistent with this role, ts mutants of *ORC2* and *ORC5* exhibit defects in plasmid stability (Micklem *et al.,* 1993; Loo

et al., 1995); these defects can be reverted by tandem addition of extra origins of replication on the plasmid (Hogan and Koshland, 1992; Loo *et al.,* 1995). This phenotype is again suggestive of a defect in the efficiency of origin use (Hogan and Koshland, 1992), a defect clearly shown by two-dimensional gel analysis of ARSs (Liang *et al.,* 1995; Loo *et al.,* 1995). Similar phenotypes have also been observed in *ORC1* (Hori *et al.,* 1996), *ORC6* (Ford and Chevalier, 1995), and *ORC3* (Loo *et al.,* 1995). All these data clearly suggest a role for ORC complexes as a DNA replication initiators, a role further supported by the genetic interactions between *ORC1–6* genes and other genes that participate in DNA replication (Dutta and Bell, 1997). However, although these results indicate that ORC is required for DNA replication, this complex is not sufficient to drive the initiation of DNA replication, as shown by the permanent binding to chromatin of ORC proteins (Aparicio *et al.,* 1997), even in cell cycle stages in which no initiation of DNA replication occurs.

Given that ORC recognizes ARSs directly and that the function of the complex is not sufficient for the initiation event to occur, it has been proposed that ORC plays its main role by serving as a landing path for proteins required for this event (Stillman, 1996). In this sense, it has been shown that ORC interacts with ARS and with Cdc6 (Liang *et al.,* 1995), an essential component of pre-RCs. Specific DNA-binding motifs have been found in ORc2 protein (Aravind and Landsman, 1998), but no single ORC subunit is capable of binding DNA. The DNA-binding activity of ORC depends on the coordinate action of Orc1–Orc5 proteins but is independent of Orc6, the only protein dispensable for DNA recognition and assembly (Lee and Bell, 1997). Protein–DNA cross-linking studies suggest that the binding of Orc1, Orc3, and Orc6 proteins, on the one hand, and the binding of Orc2, Orc4, and Orc5 proteins, on the other hand, occurs in different areas of ARSs (Lee and Bell, 1997).

The binding of ORC to ARSs depends on ATP (Rowley *et al.,* 1995). Orc1 and Orc5 proteins have potential nucleotide-binding motifs—two in Orc1 and one in Orc5 (Loo *et al.,* 1995). Mutations in the unique motif of the Orc5 protein cause growth defects, whereas mutations in the first domain of the Orc1 protein are unviable (Loo *et al.,* 1995), indicating the important role for ATP binding in ORC function. In addition to the ability of ORC to bind ATP and ARSs, the complex also works as an ATPase function mediated by the Orc1 ATP binding domain (Klemm *et al.,* 1997). This biochemical property is not needed to bind DNA since ATP remains associated with ORC–ARS complexes (Klemm *et al.,* 1997). These data indicate that ORC-dependent ATP hydrolysis occurs once the complex has been bound to ARSs and could well involve the assembly of other proteins to origins of replication (a preinitiation event), conformational changes at origins or in the associated complexes during initiation, or disassembly steps of complexes once initiation events have been triggered. Although the biochemical nature of these proteins is known, the order in which these steps may act *in vivo* is not fully understood.

2. Components of Prereplicative Complexes at Origins

Two strong lines of evidence suggested the existence of an additional protein complex involved in the control of DNA replication initiation. First, ORC was required but was not sufficient to drive the initiation of DNA synthesis at ARSs. Second, genomic analysis showed that the footprinting/imprinting at ARSs during the G_1 period just before S phase was broader than in the rest of the cell cycle. This new complex was first suggested to contain ORC and a G_1-specific factor jointly (Diffley *et al.,* 1994) and was named the pre-RC. The characterization of pre-RC components followed a biochemical approach, revealing that Cdc6 and members of the Mcm family of proteins are required for the licensing reaction (i.e., the molecular mechanisms allowing the initiation of DNA replication). Here, we summarize the evidence supporting these conclusions, the components of pre-RC, and what is known about their function in triggering DNA synthesis at eukaryotic ORIs.

a. Cdc6 Different lines of evidence have suggested a critical role for Cdc6 in the initiation of genome replication. A ts mutant of the *CDC6* gene was isolated in the original *cdc* search in *S. cerevisiae* by Hartwell (1973). Analysis of the *cdc6-1* ts allele showed that the gene function was required to initiate DNA replication (Hartwell, 1976) because G_1 cells carrying the ts mutation stop cell cycle progression during the early steps of DNA replication (Bueno and Russell, 1992; Piatti *et al.,* 1995). Cells carrying this ts mutation had elevated rates of minichromosome loss, even at semipermissive temperatures. Significantly, this defect can be reverted by tandem addition of extra origins of replication to the minichromosome (Hogan and Koshland, 1992), suggestive of origin firing defects (Hogan and Koshland, 1992), as confirmed by direct analysis of origin activity by measuring chromosomal ARS efficiency with 2D gels (Liang *et al.,* 1995). In fact, Cdc6 plays a direct role in the formation and maintenance of pre-RCs at origins *in vivo* (Cocker *et al.,* 1996; Santocanale and Diffley, 1996). This essential role is most likely exerted through the direct interaction of the Cdc6 protein with the ORC complex (Liang *et al.,* 1995; Mizushima *et al.,* 2000), presumably by increasing the binding specificity of the ORC complex to DNA (Mizushima *et al.,* 2000). The Mcm protein complex that forms part of pre-RC complexes binds ARS–ORC in a Cdc6-dependent manner (Donovan *et al.,* 1997), also indicative of the presence of Cdc6 in pre-RCs. Nevertheless, once the Mcm complex has bound to chromatin, Cdc6 is no longer required for the maintenance of pre-RCs at origins (Donovan *et al.,* 1997). All the genetic evidence obtained to date is consistent with this hypothesis concerning the role of Cdc6 in prereplicative complex formation (Liang *et al.,* 1995; Cocker *et al.,* 1996; Tanaka *et al.,* 1997; Perkins and Diffley, 1998).

Unlike ORC components, Cdc6 protein levels oscillate during the cell cycle. The presence of this protein is limited to the G_1 phase of the cell cycle (Piatti *et al.,* 1995; Detweiler and Li, 1997). This pattern of expression may well be the consequence of two different regulatory mechanisms: periodic expression of the

gene and rapid degradation of the protein at the G_1 to S phase transition. In cycling cells, *CDC6* mRNA peaks at the M/G_1 transition point (Bueno and Russell, 1992; Zwerschke *et al.,* 1994; Piatti *et al.,* 1995), whereas in cells reinitiating the cell cycle from a G_1 arrest *CDC6* is expressed late in that period, at least during the first cell cycle (Zhou and Jong, 1990; Bueno and Russell, 1992). It is accepted that *CDC6* is normally transcribed only at the exit from mitosis and that the late G_1 burst of expression is a consequence of the need to form pre-RC complexes in cells exiting a pheromone arrest (Piatti *et al.,* 1995; Dutta and Bell, 1997). In both cases, Cdc6 protein levels parallel mRNA abundance (Piatti *et al.,* 1995; Detweiler and Li, 1997). The Cdc6 protein is highly unstable (Drury *et al.,* 1997; Piatti *et al.,* 1996; Sánchez *et al.,* 1999; Calzada *et al.,* 2000) and disappears at the G_1/S transition. The rapid degradation of Cdc6 is regulated by both CDK1/Cln and CDK1/Clb complexes (Drury *et al.,* 1997; Calzada *et al.,* 2000). This CDK-mediated phosphorylation triggers a degradation pathway (Drury *et al.,* 1997) involving Cdc6 polyubiquitination (Sánchez *et al.,* 1999), most likely through the proteasome, although this detail remains to be elucidated.

The dependence on Cdc6 to form pre-RCS (Cocker *et al.,* 1996) explains why these complexes are only present during G_1 and how the genome is licensed to be replicated. CDK-dependent Cdc6 degradation is therefore one of the multiple and redundant CDK-mediated controls ensuring single rounds of replication every cell cycle (see Sections III.C and V). Cdc6 contains an ATP binding domain that consists of two separate peptide sequences referred to as the A and B Walker motifs (Walker *et al.,* 1982; Zhou and Jong, 1990; Elsasser *et al.,* 1996; Perkins and Diffley, 1998). Thus, Cdc6 is a member of the AAA^+ family of proteins (ATPases associated with cellular activities) (Patel and Latterich, 1998; Perkins and Diffley, 1998; Neuwald *et al.,* 1999). Based on studies of known ATPases, the A motif is thought to contact the triphosphate moieity, and the B motif is thought to coordinate a Mg^{2+} ion important for ATP hydrolysis (Story and Steitz, 1992). Nucleotide binding by AAA^+ proteins is believed to elicit conformational changes that promote remodeling of target protein and/or protein complexes. In this context, mutations on the Cdc6 A domain that abrogate ATP binding show that ATPase activity is required for cell viability and initiation of DNA replication (Elsasser *et al.,* 1996; Perkins and Diffley, 1998; Weinreich *et al.,* 1999; A. Calzada and Bueno, A. unpublished results). It has been shown that the ATP binding and/or hydrolysis activity of Cdc6 is required *in vivo* for Mcm complex loading on ORC–chromatin complexes (Perkins and Diffley, 1998; Weinreich *et al.,* 1999) and ORC structure modulation of DNA binding activity *in vitro* (Mizushima *et al.,* 2000), probably in a sequential process (Seki and Diffley, 2000).

b. Mcm Complex Minichromosome maintenance (MCM) genes were identified in a genetic screening of mutants defective in minichromosome maintenance (Moir *et al.,* 1982; Maine *et al.,* 1984). Six MCM genes were shown to be required for the initiation of DNA replication in budding yeast (*MCM2–7*) (Dutta and Bell, 1997).

Because of their homology the MCM gene-encoded products were grouped into a family (Chong *et al.,* 1996). The region most conserved in these proteins includes a DNA-dependent ATPase domain (Koonin, 1993). In the previously mentioned genetic screening, other MCM genes were identified. One of them, *MCM1,* encodes a transcription factor regulating the transcription of some MCM genes, *CDC6,* and other genes participating in DNA replication (see Section II.D). Another, *MCM10,* encodes a protein that shows no homology with the Mcm2–7 family, participating in genome replication as an initiation factor. It has been shown that the other isolated MCM genes are involved in chromosome segregation (Dutta and Bell, 1997).

First identified in *S. cerevisiae,* the Mcm2–7 family is conserved in all eukaryotes and, remarkably, some homologous proteins have been found in archaeabacteria (Kearsey and Labib, 1998). Despite their structural and functional conservation, each Mcm protein is essential for cell growth, initiation, and elongation steps in DNA replication (Yan *et al.,* 1993; Labib *et al.,* 2000). This observation indicates that the function of one Mcm protein cannot be substituted by that of the others. Apart from the minichromosome maintenance defect, MCM ts mutants show cell division cycle (cdc) arrest (Gibson *et al.,* 1990; Yan *et al.,* 1991). MCM mutant cells arrest at the G_1 to S phase transition or along the S phase (Yan *et al.,* 1991; Labib *et al.,* 2000). The different arrest points of these MCM mutants are consistent with defects in the initiation of DNA synthesis but also with defects in the elongation step of the replication machinery. It has been suggested that MCM mutants that arrest along S phase show this phenotype as a consequence of the incomplete replication that occurs after the initiation from a small number of replication origins (Maine *et al.,* 1984; Gibson *et al.,* 1990). Accordingly, mutants in different MCM genes show diminished frequency of replication initiation at origins as measured by 2D gel analysis of replication intermediates (Yan *et al.,* 1993; Lei *et al.,* 1996).

MCM genes are periodically transcribed along the cell division cycle, reaching maximum expression at the end of mitosis (Hennessy *et al.,* 1990; Whitebread and Dalton, 1995). However, Mcm protein levels are constant at high concentrations in proliferating cells throughout the different stages of the cell cycle (Donovan *et al.,* 1997; Young and Tye, 1997). This means that Mcm proteins are constantly in excess relative to replicators and the other initiators, ORC and Cdc6 (Lei *et al.,* 1996). Nevertheless, despite their constant presence they are not available for pre-RC formation except in the G_1 phase (Piatti *et al.,* 1996; Aparicio *et al.,* 1997; Tanaka *et al.,* 1997; Labib *et al.,* 1999). In *S. cerevisiae,* it was thought that the subcellular location of the Mcm proteins changed along the cell cycle (Dutta and Bell, 1997). It was also reported that Mcm proteins were nuclear during G_1 but became cytoplasmic when cells initiated S phase (Hennessy *et al.,* 1990; Yan *et al.,* 1993; Whitebread and Dalton, 1995).

This hypothesis was inconsistent with the suggested role of the Mcm complex as the *S. cerevisiae* helicase, playing a role during the elongation step of DNA

synthesis (Tye, 1999; Labib *et al.,* 2000), and it is difficult to reconcile with the constant requirement of the Mcm proteins throughout the S phase (Labib *et al.,* 2000). Further work indicated that Mcm proteins are constantly nuclear both in yeasts and in metazoans (Thommes *et al.,* 1992; Todorov *et al.,* 1994; Kimura *et al.,* 1994; Maiorano *et al.,* 1996; Young and Tye, 1997). These contradictory results might be explained if the Mcm nuclear signal is lost through binding of the Mcm complexes to chromatin in a cellular context in which the cytoplasmic Mcm proteins, and hence their signal, predominate (Tye, 1999). However, the nuclear import/export mechanism does not appear to be the control mechanism by which the association of the Mcm complex to chromatin is regulated (Young and Tye, 1997; Walter *et al.,* 1998; Tye, 1999). In fact, phosphorylated Mcm7 in G_2 is unable to bind chromatin, even in the presence of chromatin-bound ORC–Cdc6 complexes (Tanaka *et al.,* 1997), suggesting that the affinity to bind the other initiators at origins depends on the Mcm phosphorylation status. It is generally accepted that regulation of the chromatin binding of Mcm proteins is part of the redundant mechanism by which cells restrict DNA replication to a single round in S phase (Tye, 1999; Labib *et al.,* 2000). These proteins are excluded from the nucleus by a CDK-dependent mechanism since both CDK1/Cln and CDK1/Clb5,6 regulate their subcellular localization (Labib *et al.,* 1999; Nguyen *et al.,* 2000) in two different ways—by a mechanism simply regulated by CDK when a functional Cdc6 is absent or by a mechanism regulated by CDK but dependent on S phase initiation when a functional Cdc6 is present (Nguyen *et al.,* 2000). Taking into account the fact that Mcm proteins are present in G_1 nuclei in excess with respect to other initiators, including origins of replication (Lei *et al.,* 1996), it is possible that chromatin-free Mcms might be excluded from the nucleus by CDK activity, while chromatin-bound Mcms would require passage through S phase to be released from DNA (perhaps in a Cdc6 degradation-dependent manner) since they complete their elongation function during DNA synthesis (Tye, 1999).

In vitro studies from yeast and humans suggested that a hexameric complex can be formed by the association of all six Mcm proteins, although smaller subcomplexes formed by three or four different Mcm protein subunits have also been observed (Ishimi, 1997; Tye, 1999). The formation of a similar complex *in vivo* is consistent with the coimmunoprecipitation of all six proteins with antibodies raised against one of them (Lei *et al.,* 1996; Dalton and Hopwood, 1997). Chromatin cross-linking assays clearly suggest that the hexameric complex is recruited to replication origins before the onset of DNA synthesis (Tanaka *et al.,* 1997). The conserved ATPase motif shared by members of the MCM family suggests that the hexameric complex may be associated with ATPase and/or helicase activity. Consistent with this hypothesis, *in vitro* and *in vivo* evidence from *S. pombe* or mouse Mcm proteins, in the first case (You *et al.,* 1999), and human Mcm proteins in the second (Ishimi, 1997), has been obtained, indicating that the hexameric complex could function as a helicase in the elongation phase of DNA synthesis. In the case of *Saccharomyces,* the complex is bound to replicators in G_1 and nonorigin

DNA during S phase (Aparicio *et al.,* 1997). Further confirmation of the "helicase hypothesis" derives from the use of rapidly degradable Mcm–Degron mutants showing that the degradation of individual Mcm complex components after initiation of DNA replication completely and irreversibly blocks the progression of replicative forks (Labib *et al.,* 2000).

c. Mcm10 The *MCM10* (*DNA43*) gene was characterized as being essential for the initiation of DNA replication (Merchant *et al.,* 1997). Isolated in the same screening that allowed the identification of the other MCM genes, it shares many of the phenotypes of the ts mutants of the *MCM2–7* family, despite sharing no sequence homology with them. *MCM10-1* ts mutants show defects in the efficiency of origin firing, both chromosomal and plasmid located (Merchant *et al.,* 1997; Homesley *et al.,* 2000). However, these defects can also be found in mutants of genes not directly related to initiation of DNA replication, such as *CDC14* (Hogan and Koshland, 1992; Noton and Diffley, 2000; A. Calzada and A. Bueno, unpublished results). Direct evidence of the role of Mcm10 in DNA replication derives from the Degron strategy, which shows that the protein is required in DNA synthesis for the elongation step (Merchant *et al.,* 1997), although in this case the pause caused after Mcm10–Degron proteolysis occurs when replication forks pass through origins of replication that have not been fired (passively replicated) (Merchant *et al.,* 1997). Mcm10 binds to ARSs independently of ORC, and chromatin binding of both is required for Mcm7 recruitment to origins during the G_1 phase (Homesley *et al.,* 2000). It has been suggested that Mcm10 participates not only in the assembly but also in the disassembly of pre-RCs (Merchant *et al.,* 1997; Homesley *et al.,* 2000). The promoter of the *MCM10* gene contains MCB elements (Toyn *et al.,* 1995), and transcription of the gene is in fact controlled by Mbp1 and Swi6 factors (Toyn *et al.,* 1995). The Mcm10 protein is located nuclearly throughout the cell cycle (Merchant *et al.,* 1997). The precise role of Mcm10 in DNA replication and how its function is controlled, most likely by the cell cycle machinery and limited to the G_1 phase, remain to be elucidated.

d. Cdc45 Cdc45 has recently been identified as an initiation factor (Zou *et al.,* 1997) but is also involved in DNA repair mechanisms (Hopwood and Dalton, 1996). *CDC45* ts mutants arrest at the G_1/S boundary or late S phase, depending on the allele (Hennessy *et al.,* 1991; Moir *et al.,* 1982). These mutants show initiation of DNA replication defects such as the classic single ARS plasmid loss defect that can be reverted with additional ARSs (Hardy, 1997), a high rate of chromosome loss (Hennessy *et al.,* 1991), and defects that relate the protein to the elongation of DNA replication (Reid *et al.,* 1999).

Direct interaction between the Cdc45 and Mcm proteins has been reported (Hopwood and Dalton, 1996; Zou *et al.,* 1997; Zou and Stillman, 1998), which suggests that the role of Cdc45 in replication initiation must be coupled to the action of the Mcm complex at origins (Zou and Stillman, 1998). After START, the

TABLE II
Homologs of DNA Synthesis Initiator Genes in Eukaryotes[a]

S. cerevisiae	*S. pombe*	*C. elegans*	*D. melanogaster*	*X. laevis*	*M. musculus*	*H. sapiens*
ORC1	*orp1*$^{+1,2,3}$	*CeORC1*[18]	*ORC1*[19]	*XIORC1*[30]	*Orc1* [39]	*ORC1L*[3, 48]
ORC2	*orp2*$^{+}$/*cdc30*$^{+4}$	*orc-2*[18]	*ORC2*[20]	*XIORC2*[31]	*ORC2*[40]	*ORC2L*[3, 40]
ORC3	*orc3*$^{+5}$	—	*latheo*[21]	—	*ORC3*[41]	*LAT*/*ORC3*[49]
ORC4	*orc4*$^{+5-7}$	—	*ORC4*[22]	*XIORC4*[32]	*ORC*[41]	*HsORC4*[32, 50]
ORC5	*orc5* $^{+5,8}$	—	*DmORC5*[20]	*XIORC5*[32]	*ORC5*[41]	*ORC5L*[8, 32, 60]
ORC6	*orc6*$^{+5}$	—	—	—	—	*ORC6*[23]
CDC6	*cdc18*$^{+9}$	*CeCDC6*[18]	—	*XICDC6*[33]	*Cdc6*[42]	*CDC18L*[51]
MCM2	*cdc19*$^{+}$/*nda1*$^{+10,11}$	Five MCM related	*Mcm2*[24]	*xMCM2*[34]	*Mcmd2* / *B1*[43]	*BM28*[52]
MCM3	*mcm3*$^{+12}$	proteins	*Mcm3*[25]	*XIMCM3*[35]	*P1* / *Mcmd* [44]	*P1*[35, 44]
MCM4 / *CDC54*	*cdc21*$^{+13}$	described by	*dpa*[26]	*XIMCM4*[36]	*Mcmd4* / *mCdc21*[45]	*HsMCM4*[53]
MCM5 / *CDC46*	*nda4*$^{+11}$	the *C. elegans*	*Mcm5*[27]	*xCDC46*[34]	*mCDC46* / *Mcmd5*[45]	*HsMCM5*[54]
MCM6	*mis5*$^{+14}$	Sequencing	*Mcm6*[25]	—	*Mcmd6* / *Mmis5*[43]	*HsMCM6*[55]

MCM7 / CDC47	*mcm7*$^{+6}$	Consortium[18]	*Mcm7*[25]	*XMCM7*[37]	*mCDC47*[46]	*HsCDC47*[56, 57]
MCM10/DNA43	*cdc23*$^{+15}$	—	—	—	—	*HsMCM10*[58]
CDC45	*sna41*$^{+16}$	*CeCDC45*[18]	*DmCDC45*[28]	*XlCDC45*[38]	*CDC45L*[47]	*CDC45L*[47]
—	*cdt1*$^{+17}$	—	—	*XCDT1*[29]	—	*Cdt1*[59]

[a] The original name for each gene is indicated. However, currently each protein is known with the name of the family preceded by an indication of the specie (e.g., *P1* is known as *HsMCM3*).

1, Grallert and Nurse (1996); **2,** Muzi-Falconi and Kelly (1995); **3,** Gavin *et al.* (1995); **4,** Leatherwood *et al.* (1996); **5,** Moon *et al.* (1999); **6,** the *Schizosaccharomyces pombe* Genome Sequencing Project, http : //www.sanger.ac.uk/Projects/S_pombe/; **7,** Chuang and Kelly (1999); **8,** Ishiai *et al.* (1997); **9,** Kelly *et al.* (1993); **10,** Forsburg and Nurse (1994); **11,** Miyake *et al.* (1993); **12,** Sherman and Forsburg (1998); **13,** Maiorano *et al.* (1996); **14,** Takahashi *et al.* (1994); **15,** Aves *et al.* (1998); **16,** Miyake and Yamashita (1998); **17,** Hofman and Beach (1994); **18,** the *C. elegans* Sequencing Consortium (1998); **19,** Pak *et al.* (1997); **20,** Gossen *et al.* (1995); **21,** Boynton and Tully (1992); **22,** Chesnokov *et al.* (1999); **23,** Dhar and Dutta (2000); **24,** Treisman *et al.* (1995); **25,** Ohno *et al.* (1998); **26,** Feger *et al.* (1995); **27,** Su *et al.* (1997); **28,** Loebel *et al.* (2000); **29,** Maiorano *et al.* (2000); **30,** Rowles *et al.* (1996); **31,** Carpenter *et al.* (1996); **32,** Tugal *et al.* (1998); **33,** Coleman *et al.* (1996); **34,** Miyake *et al.* (1996); **35,** Kubota *et al.* (1995); **36,** Hendrickson *et al.* (1996); **37,** Romanowski *et al.* (1996a); **38,** Mimura and Takisawa (1998); **39,** Zisimopoulou *et al.* (1998); **40,** Takahara *et al.* (1996); **41,** Springer *et al.* (1999); **42,** Hateboer *et al.* (1998); **43,** Kimura *et al.* (1996); **44,** Thommes *et al.* (1992); **45,** Kimura *et al.* (1995); **46,** Takizawa *et al.* (1995); **47,** Shaikh *et al.* (1999); **48,** Eki *et al.* (1996); **49,** Pinto *et al.* (1999); **50,** Quintana *et al.* (1997); **51,** Williams *et al.* (1997); **52,** Todorov *et al.* (1994); **53,** Musahl *et al.* (1995); **54,** Hu *et al.* (1993); **55,** Harvey *et al.* (1996); **56,** Schulte *et al.* (1996); **57,** Kiyono *et al.* (1996); **58,** Izumi *et al.* (2000); **59,** Wohlschelegel *et al.* (2000); **60,** Quintana *et al.* (1998).

Cdc45 protein associates with chromatin and remains bound in late G_1 and during S phase to dissociate gradually in late S phase or G_2 (Zou and Stillman, 1998). Cdc45 binding to chromatin is negatively regulated by CDK1/Clb kinases and positively regulated by Cdc6 and Mcm2 (Zou and Stillman, 1998). Additionally, Cdc45 loading on chromatin requires Cdc7/Dbf4 activity (Zou and Stillman, 1998), and in mutants defective for *CDC7* the binding of Cdc45 is not sufficient to convert pre-RCs to post-RCs (Zou and Stillman, 1998). The timing of association of Cdc45 to pre-RCs and the activation of CDK1/Clb complexes suggest that this initiator protein is recruited after the assembly of pre-RCs at origins and after CDK1/Clb activation occurs. Because the action of Cdc7 (see Sections III.C and IV.B) is required for the actual entry into S phase, the action of Cdc45 must be temporally coincident with the initiation of DNA replication.

As S phase progresses, particularly from early to late S phase, the Cdc45 protein changes its location from replication origins (ARSs) to nonorigin chromatin regions, suggesting that, like the Mcm complex, Cdc45 may also be associated with the elongation machinery (Aparicio *et al.,* 1997).

3. Possible Additional Initiators

a. Cdt1 This member of the initiator family has been cloned and characterized in *S. pombe* (Hofmann and Beach, 1994). Comparison with the genome of the budding yeast *S. cerevisiae* reveals that there is no obvious homolog of this gene, although *Xenopus* and *Homo sapiens* homologs have been described (Table II). The *S. pombe cdt1*$^+$ gene was cloned as a cdc10-dependent transcript (Hofmann and Beach, 1994). Cdc10 is the fission yeast homolog of budding yeast Swi6 (see Section II.D). Null *cdt1* mutants are unviable and their cells attempt to undergo mitosis without having replicated their DNA (Hofmann and Beach, 1994). Recent studies showed that the Cdt1 protein localizes at the nucleus in G_1 and the early S phase (Nishitani *et al.,* 2000) and that it interacts physically with Cdc18, the *S. pombe* homolog of Cdc6, as shown by coimmunoprecipitation assays (Nishitani *et al.,* 2000).

b. Mec1 and Rad53 *MEC1* and *RAD53* are checkpoint genes that regulate the rate of progression through S phase in budding yeast in response to DNA damage caused by radiation or methylating agents (Paulovich and Hartwell, 1995). Recently, it was suggested that these genes play a role in the regulation of the activation of the origins of replication that initiate DNA synthesis late during S phase (late origins). In particular, the observation in budding yeast that hydroxyurea, a drug that blocks S phase by inhibiting the progression of replication forks from early firing origins, also inhibits firing from late origins led to experiments that indicated that late origins were maintained in a initiation-competent prereplicative state for extended periods (Santocanale and Diffley, 1998). Significantly, the inhibition of late origin firing was defective in yeast with mutations in *MEC1* and

RAD53. Additionally, analysis of the timing of activation of replication origins in chromosome VI of *S. cerevisiae* revealed that they initiated replication in a sequential manner during S phase of the cell cycle (Friedman *et al.,* 1997; Yamashita *et al.,* 1997). Remarkably, a mutation of the *RAD53* gene resulted in late origins becoming early replicating, suggesting that the Rad53 protein is directly involved in the timing of activation of late origins during a normal cell cycle, most likely forming part of the surveillance mechanism of S phase progression of *S. cerevisiae* cells (Shirahige *et al.,* 1998). A direct interaction of these proteins with pre-RCs remains to be elucidated, although data suggest that Mec1 and Rad53 proteins may form part of pre-RCs at late origins. Alternatively, both proteins may inhibit the regulators that activate origins (see Section III.C) in a pre-RC interaction-independent mechanism. Recent *in vitro* studies have shown that the kinase encoded by *RAD53* is able to inhibit, by phosphorylation, the Cdc7/Dbf4 kinase, suggesting that Rad53 controls the initiation of chromosomal DNA replication by regulating the Cdc7 kinase complex (Kihara *et al.,* 2000). Homologs of both *MEC1* and *RAD53* have been described throughout the eukaryotic evolutive scale (Costanzo *et al.,* 2000).

4. Conservation of Prereplicative Complex Components from Yeast to Mammals

Given the common nature of the processes involved in the genome replication that eukaryotic cells face in each S phase, elements and proteins regulating and/or participating in the initiation of chromosomal DNA replication are likely to be conserved along the evolutive scale. Initiation of DNA replication in cells of *Xenopus, Drosophila,* and *H. sapiens* (examples of multicellular systems) begins at specific chromosomal locations, at individual replication origins, in a comparable way to yeast (Antequera and Bird, 1999; DePamphilis, 1999). Furthermore, during the past decade most, if not all, of the proteins that participate in DNA replication initiation in the budding yeast have been identified, and in some cases characterized, in all eukaryotic models. Homologs of *S. cerevisiae* initiation proteins have been identified in yeast, other fungi, plants, nematodes, flies, frogs, and mammals (Table II). These homologs include all six origin recognition proteins (ORC), the six minichromosome maintenance proteins, the initiator protein responsible for the loading of MCM complex (Cdc6), the Cdc7/Dbf4 kinase complex, and Cdc45 (Table II). Although the nature, organization, and functionality of the replicators of multicellular eukaryotes remain unclear (Antequera and Bird, 1999; DePamphilis, 1999), the conservation of the initiators suggests the maintenance of a similar, if not identical, mechanism of DNA replication initiation in all eukaryotes. Consistent with this hypothesis, it has been shown that several of these homologs are required for DNA replication in *Drosophila, Xenopus, Mus musculus,* and *H. sapiens* (Table II). Nevertheless, despite this degree of conservation, the specific nature of the events leading to initiation of genome replication in multicellular organisms may differ in details from one system to another, as suggested by the role of

the ORC complex in the mechanism ensuring prereplicative complex assembly at origins in hamster cells (Natale *et al.,* 2000).

C. Regulators of DNA Replication at Origins

The formation of pre-RCs at origins of replication is an essential step for the initiation of DNA replication during S phase, but the assembly of these molecular macrostructures is insufficient to ensure that DNA synthesis will start at any given origin of replication (Dutta and Bell, 1997). During the past decade, it has become clear that the initiation of genome replication also requires the activity of other components, particularly protein kinases and their cyclin partners, including both Cdc28/Clb (CDK) and Cdc7/Dbf4, also known as DDK (Dbf4-dependent kinase) (Johnston *et al.,* 1999). Activation of both kinases at the end of G_1 is thought to be the event that controls the initiation of DNA synthesis at replicators and hence the transition from G_1 to S phase. For many years, it has been known that the DDK complex interacts physically with pre-RCs at origins to exert its function (Dowell *et al.,* 1994), whereas the interaction or the lack of direct interaction of CDK complexes remains unclear. For these reasons, we consider DDK complexes as *in situ* activators and CDK as transactivators of the initiation reaction leading to DNA synthesis from origins of replication.

1. *In Situ* Activators: Cdc7/Dbf4 or DDK

Cdc7/Dbf4 or DDK complexes are formed by a catalytic subunit, Cdc7, and a cyclin-like subunit, Dbf4. The *CDC7* gene was identified as a gene required for the early steps of DNA replication in *S. cerevisiae* (Hartwell, 1973, 1978) and encodes a protein with similarities to protein kinases (Patterson *et al.,* 1986). The Cdc7 protein has kinase activity *in vitro* (Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991) and activity fluctuates along the cell division cycle, peaking at the G_1 to S phase transition (Jackson *et al.,* 1993; Yoon *et al.,* 1993). In the absence of Cdc7, budding yeast cells arrest prior to DNA replication with high levels of Cdc28/Clb kinase (Amon *et al.,* 1992). It has been suggested that the *CDC7* execution point is around the end of G_1 and the beginning of S phase because Cdc7/Dbf4 kinase activity is elevated when S phase-promoting CDKs are turned on (Nougarede *et al.,* 2000). Nevertheless, its activity is required throughout S phase (Bousset and Diffley, 1998; Donaldson *et al.,* 1998).

To be active, Cdc7 requires a positive regulator—Dbf4 (Jackson *et al.,* 1993; Kitada *et al.,* 1993). *Saccharomyces cerevisiae* cells bearing mutations in *DBF4* show a terminal phenotype that is indistinguishable from *cdc7* ts mutants (Johnston and Thomas, 1982; Solomon *et al.,* 1992). The combination of mutations in *DBF4* and *CDC7* results in cell inviability (Kitada *et al.,* 1993), the so-called "synthetic

lethality phenotype," suggesting physical interaction and/or a common function *in vivo*. Consistently, the Dbf4 and the Cdc7 proteins interact *in vivo* (Dixon and Campbell, 1997). Additionally, Cdc7-dependent kinase activity is thermosensitive when immunoprecipitated from *cdc7* or *dbf4* ts mutant cells (Jackson *et al.,* 1993). Thus, Dbf4 is an essential subunit of the Cdc7 kinase activity and therefore, by analogy to cyclin-dependent kinases (CDKs), Dbf4 is considered to be the cyclin-like protein of the Cdc7–kinase complex DDK (Johnston *et al.,* 1999). In addition, DDK interacts with replication origins through its Dbf4 moiety, which has an ORC binding site (Dowell *et al.,* 1994). Consistent with this, genetic analyses have shown that DDK interacts with ORC and Mcm protein complexes (Fox *et al.,* 1995; Loo *et al.,* 1995; Hardy *et al.,* 1997; Lei *et al.,* 1997), although it remains unknown through which pre-RC components DDK binds. Identification of the targets of DDK is one of the essential aims for understanding the molecular basis of the DNA replication initiation mechanisms. Given that both the Dbf4–origin interacting domain and the Cdc7–Dbf4 interacting domain are essential for the role of the DDK complex at S phase, it has been proposed that Dbf4 acts to recruit Cdc7 to replication origins (Dowell *et al.,* 1994).

It is thought that DDK complexes are required throughout S phase since Cdc7 appears to play a direct role in the firing of replication origins during S phase. Consistent with this hypothesis, it has been shown that the Cdc7 kinase is responsible for *in situ* activation of DNA replication at early origins at the beginning of S phase and late origins during S phase (Bousset and Diffley, 1998; Donaldson *et al.,* 1998). Probable substrates of the DDK are replication initiation components of pre-RC complexes. *In vitro* assays have shown that the Cdc7/Dbf4 complex phosphorylates Mcm2–4 and Mcm6 but not Mcm5 and Mcm7 (Lei *et al.,* 1997). Additionally, both genetic and biochemical data support the idea that DDK phosphorylates the Mcm2 protein *in vivo* (Lei *et al.,* 1997). Also relevant to the role of Cdc7/Dbf4 in the Mcm complex, it has been shown that *MCM5* mutants bypass the need for DDK in DNA replication (Hardy *et al.,* 1997). Recently, it has been reported that the Cdc45 protein is phosphorylated by DDK *in vitro* (Nougarede *et al.,* 2000). Together, these data have led to the suggestion that DDK could activate the helicase activity of the Mcm complex at origins of replication, thus promoting the first steps toward elongation events in DNA synthesis (Tye, 1999). Finally, it has been suggested that DDK-dependent Mcm phosphorylation could account for the irreversible inactivation of some Mcm complex components, thus playing a role in preventing rereplication (Tye, 1999), although nuclear exclusion of Mcm proteins appears to be controlled by CDK1/Clns and CDK1/Clbs complexes (Labib *et al.,* 1999; Nguyen *et al.,* 2000).

DDK activity is regulated in a cell cycle-regulated manner, reaching a peak at the G_1/S transition (Jackson *et al.,* 1993; Yoon *et al.,* 1993). *CDC7* transcript and Cdc7 protein levels remain constant along the cell cycle. Cdc7 is an *in vivo* phosphoprotein (Yoon and Campbell, 1991) detail that may be important for regulation since Cdc7 phosphorylation coincides with the activation of DDK-dependent

TABLE III
Conservation through the Eukaryotic Evolutive Scale of Regulators of DNA Replication Initiation at Origins

	S. cerevisiae	*S. pombe*	*D. melanogaster*	*X. laevis*	*M. musculus*	*H. sapiens*
DDK	*CDC7*	*hsk*$^{+1,2}$	—	*XICDC7*[8]	*muCDC7*[10]	*HuCDC7*[8,12]
	DBF4	*him1*$^{+}$/*dfp1*$^{+3}$	*chiffon*[6]	—	*muASK*[11]	*ASK*[11,13]
CDK	*CDC28*	*cdc2*$^{+4}$	*cdc2*[7]	*Cdk2*[9]	—	*Cdk2*[14]
cyclin	*CLB5*	*cig2*$^{+5}$	*cycE*[7]	*cycA*[9]	—	*cycE*[14]
	CLB6			*cycE*[9]		

1, Masai *et al.* (1995); **2,** Brown and Kelly (1998); **3,** Takeda *et al.* (1999); **4,** MacNeill *et al.* (1991); **5,** Mondesert *et al.* (1996); **6,** Landis and Tower (1999); **7,** Knoblich *et al.* (1994); **8,** Sato *et al.* (1997); **9,** Strausfeld *et al.* (1996); **10,** Kim *et al.* (1998); **11,** Lepke *et al.* (1999); **12,** Jiang and Hunter (1997); **13,** Kumagai *et al.* (1999); **14,** Krude *et al.* (1997).

kinase activity (Yoon *et al.,* 1993). Consistent with this, the dephosphorylation of the Cdc7 protein *in vitro* reduces the DDK kinase activity (Dutta and Bell, 1997). The binding of Dbf4 to Cdc7 regulates DDK-associated activity. The *DBF4* gene is cell cycle regulated, with its transcript reaching a peak at the G_1/S transition, coincident with the maximum for DDK activity (Chapman and Johnston, 1989). Moreover, the Dbf4 protein is unstable; the protein begins to accumulate before the initiation of S phase, remains high until late mitosis, and then declines at around the metaphase to anaphase transition (Cheng *et al.,* 1999; Ferreira *et al.,* 2000). Thus, Dbf4 persists longer than DDK-associated activity, which gradually declines from early S phase. These data indicate that there should be an extra level of control regulating DDK activity. Finally, Cdc7 and Dbf4 homologs have been characterized, at least in *S. pombe, Xenopus, M. musculus,* and *H. sapiens* (Table III).

2. Transactivators: CDK1/Clb5 and Clb6 (S-CDKs)

Direct evidence for the role of CDKs at the initiation of DNA replication derives from studies using *in vitro* SV40 replication assays (Roberts and D'Urso, 1988). These studies showed that CDK activity increases the efficiency of unwinding of the origin of DNA replication (Roberts and D'Urso, 1988; D'Urso *et al.,* 1990; Dutta and Bell, 1997). Genetic studies using conditional alleles indicated that *CDC28* is needed in *S. cerevisiae* for the initiation of S phase (Hartwell *et al.,* 1974). It is known that the key cyclins for CDK in the activation of DNA replication are Clb5 and Clb6, which appear at the onset of S phase (Epstein and Cross, 1992; Richardson *et al.,* 1992; Schwob and Nasmyth, 1993). These CDKs are named S-CDKs after S phase promoting CDKs. Mutation of *CLB5* and/or *CLB6* genes results in delayed S phase progression. Nevertheless, any other B-type cyclin of budding yeast can replace them in S phase initiation, as deduced from the genetic evidence showing that neither of them is essential for cell viability (Schwob and

Nasmyth, 1993; Schwob *et al.,* 1994). All these data suggest that CDK1/Clb5 and Clb6 play a general role in the activation/initiation of DNA replication. Moreover, evidence pointing to the dependence on CDK1/Clb5 for the activation of late replication origins (Donaldson *et al.,* 1998) suggests that CDKs may also act directly at origins.

Activation of S-CDKs in late G_1 is a tightly regulated event, both transcriptionally and posttranslationally (see Section II.B). *CLB5* and *CLB6* mRNAs are synthesized in G_1 by a MBF-dependent mechanism (Epstein and Cross, 1992; Amon *et al.,* 1993; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993), but even these newly formed CDK1/Clb5 and Clb6 complexes are inactive until the end of G_1 (see Section III.C.3). If it is assumed that the initiation step of DNA replication is regulated by S-CDKs acting directly on origins (which is debatable), the pre-RCs complexes formed at replicators must be the targets for CDK1/Clb5,6 phosphorylating activity. This phosphorylation could account both for the activation of replication at origins and for the irreversible changes in some pre-RC components that prevent rereplication (see Sections IV and V).

Probable substrates of S-CDKs are the replication initiation components of pre-RC complexes. It is known that Cdc6 proteolysis is cell cycle regulated directly by both CDK1/G_1 and B-type cyclins (Calzada *et al.,* 2000; Drury *et al.,* 2000) and that this initiator protein is an *in vitro* substrate for CDK1/Clb5 (Elsasser *et al.,* 1996). Some of the ORC components contain putative CDK phosphorylation sites. In particular, Orc6 is phosphorylated as cells enter S phase, and substitution of Orc6 CDK-phosphorylatable amino acid residues deregulates association of this protein with the ORC complex (Liang and Stillman, 1997). Finally, as mentioned previously, nuclear localization of some Mcm proteins is regulated by both CDK1/Clns and CDK1/Clbs complexes (Labib *et al.,* 1999; Nguyen *et al.,* 2000). The consequences and probable role of S-CDK-mediated phosphorylation of pre-RC components are discussed later. Although it has been shown that CDK1/Clbs form complexes with some initiators *in vitro* and *in vivo,* as is the case for Cdc6 (Elsasser *et al.,* 1996; Calzada *et al.,* 2000), to our knowledge it remains to be elucidated whether CDK complexes are actually bound to pre-RCs components at origins. Proteins functionally equivalent to Cdc28, Clb5, and Clb6 have been identified and characterized, at least in *S. pombe, Xenopus, M. musculus,* and *H. sapiens* (Table III).

3. Inhibitors

CDK1/Clb5 and Clb6 complexes are inactive until the end of G_1 (Donaldson *et al.,* 1998; Schwob *et al.,* 1994). CDK1/Clb5 and Clb6-associated kinase activity is negatively regulated up to the G_1 to S phase transition by specific inhibitors: Sic1, which prevents S-CDKs activation until its SCF-dependent ubiquitin-tagged degradation mediated by the proteasome (Mendenhall, 1993; Schwob *et al.,* 1994; Feldman *et al.,* 1997; Skowyra *et al.,* 1997; Verma *et al.,* 1997), and Hct1, which

targets Clb cyclins for degradation in an APC-dependent manner from the end of mitosis to the end of G_1 (Alexandru *et al.,* 1999; Zachariae and Nasmyth, 1999). Both SCF and APC and their role in S phase initiation are discussed later.

IV. Initiation of DNA Replication in Yeast

Chromatin exists in two replication states throughout the cell division cycle. The first is a replication-noncompetent state, coincident with post-RCs at origins, from the beginning of S phase until the end of mitosis. The second is a replication-component state, from late mitosis to the initiation of S phase (coincident with G_1), during which pre-RCs are detected at origins of replication (Diffley *et al.,* 1994) (Fig. 11). These temporal cell cycle-regulated states of chromatin provide the mechanism for restricting DNA synthesis to a single round in each cell division. To understand S phase regulation, it is essential to know the composition and the role of the post- and pre-RC components, but it is also of critical importance to comprehend how eukaryotic cells regulate the transition between the two states. In *S. cerevisiae,* the formation of pre-RCs at origins allows the initiation of DNA synthesis by rendering chromatin competent for replication. The initiation event carries with it the transition from pre-RCs to post-RCs, a transition that involves both the opening of DNA strands and the beginning of DNA synthesis but that also prevents a new initiation event by rendering the chromatin noncompetent for replication until cells have successfully completed cell division. These cyclic transitions proceed in a timely manner, regulated by three kinase complexes: two S-CDK complexes (CDK1/Clb5 and CDK1/Clb6) and DDK (Fig. 11).

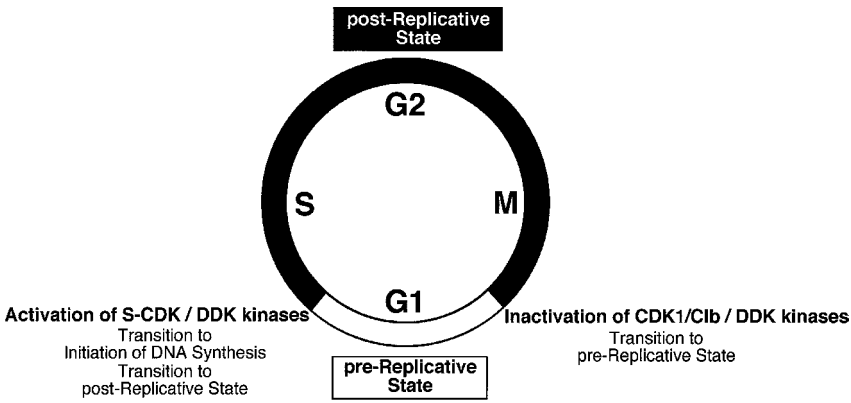


FIG. 11 The alternance between postreplicative and prereplicative states of chromatin explains how origins of replication are regulated to fire only once each cell cycle.

Next, we summarize the details of how these events are organized temporally and the evidence supporting the mechanism(s) underlying the regulatory processes, explaining the current model/view of the initiation of DNA replication.

A. M to G_1 Transition and Initiation of the S Phase

When cells exit from mitosis, under appropriate conditions they undergo a new round of cell division. Exiting mitosis is a cell cycle event characterized by the inactivation of mitotic CDKs by a two-step mechanism controlled sequentially by Cdc20 (Yeong *et al.,* 2000) and Cdc14 (Visintin *et al.,* 1998). As discussed previously, this inactivation also allows the genome to be competent for replication because it is in the absence of CDK activity when prereplicative complexes are formed at origins of replication (Diffley *et al.,* 1994; Dahmann *et al.,* 1995). Thus, the Cdc20- and Cdc14-dependent inactivation of mitotic CDKs controls the M to G_1 transition and the post- to prereplicative complex transition, regulating not only the end of the cell division but also the formation of the complexes required for the initiation of genome replication of the next cycle.

1. Inactivation of Mitotic CDKs: Removing the Block on Pre-RC Assembly

The inactivation of CDK at the exit from mitosis, in addition to other processes at the mitotic exit (Zachariae and Nasmyth, 1999; Tyers and Jorgensen, 2000), is also required for removing the CDK-mediated block on prereplicative complex assembly at origins of replication. Cdc20 and Cdc14 proteins induce inhibition of mitotic CDKs and degradation of mitotic cyclins in a APC/proteasome-specific manner (Fig. 12A). Proteolysis of mitotic Clb cyclins imposes a direction to these events that must be followed because it is irreversible.

The multisubunit APC particle is necessary for passage through anaphase, exit from mitosis, and maintenance of G_1 (Zachariae and Nasmyth, 1999; Tyers and Jorgensen, 2000). Cell cycle regulation of APC derives from its periodic association with Cdc20 and Hct1/Cdh1, two members of the WD40 family of proteins that presumably recognize the destruction box found in APC substrates (Visintin *et al.,* 1997). APC is first activated by Cdc20 at the metaphase to anaphase transition and is then activated by Hct1/Cdh1 from anaphase until the end of the G_1 phase of the next cell cycle (Zachariae and Nasmyth, 1999). The substrates of APC/Cdc20 include Pds1, Clb2, Clb3, and Clb5 (Shirayama *et al.,* 1999; Zachariae and Nasmyth, 1999; Yeong *et al.,* 2000). APC/Hct1 controls Clb2 proteolysis (Schwab *et al.,* 1997). Cdc20-dependent APC activity is controlled by CDK1/Clb kinases that directly phosphorylate APC subunits (Tyers and Jorgensen, 2000). APC/Hct1 is not activated before cells complete anaphase because CDK-dependent phosphorylation of Hct1 prevents its binding to the APC particle (Zachariae *et al.,* 1998;

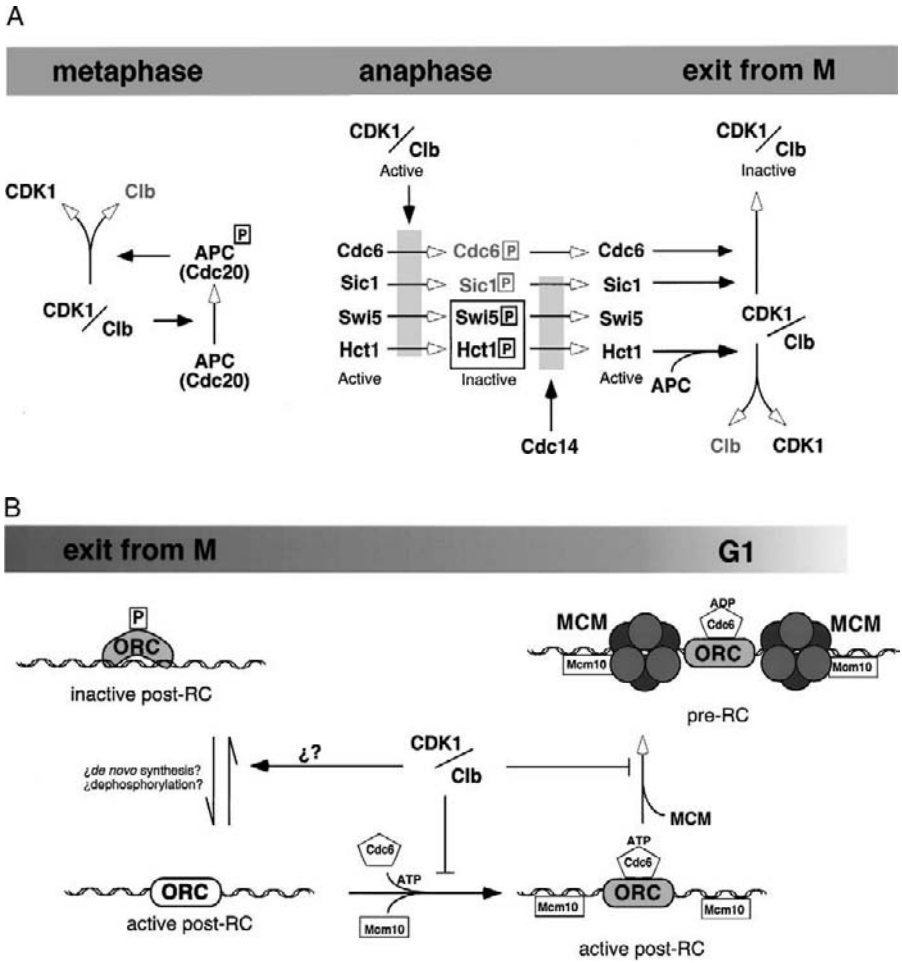


FIG. 12 (A) Exit from mitosis requires the inactivation of CDK1/Clb complexes, a prerequisite for prereplicative complex formation at origins of replication. This figure is a schematic drawing of the molecular events involved in CDK inactivation at the M to G_1 boundary. A protein network, in which Cdc6 participates, cooperates to inactivate the CDK complex by direct inhibition (Sic1 and to a lesser extent Cdc6) or by inducing Clb–cyclin proteolysis (Hct1/APC). Cdc6 dephosphorylation by Cdc14 is purely speculative. Gray area indicates proteins targeted for proteolysis. (B) The inactivation of mitotic CDK1/Clb complexes allows the assembly of prereplicative complexes at origins of replication. After the decrease in CDK1-associated kinase activity, pre-RCs are assembled at origins by a hierarchical multiple-step mechanism initiated by Cdc6 binding to post-RCs. (C) Initiation of DNA synthesis from origins of replication is regulated by the assembly of pre-RCs and CDK1/Clb5,6 DDK-mediated activation. Activation of the initiation complex results in the DNA unwinding event that allows the binding of the elongation machinery, leaving the origins in their postreplicative state.

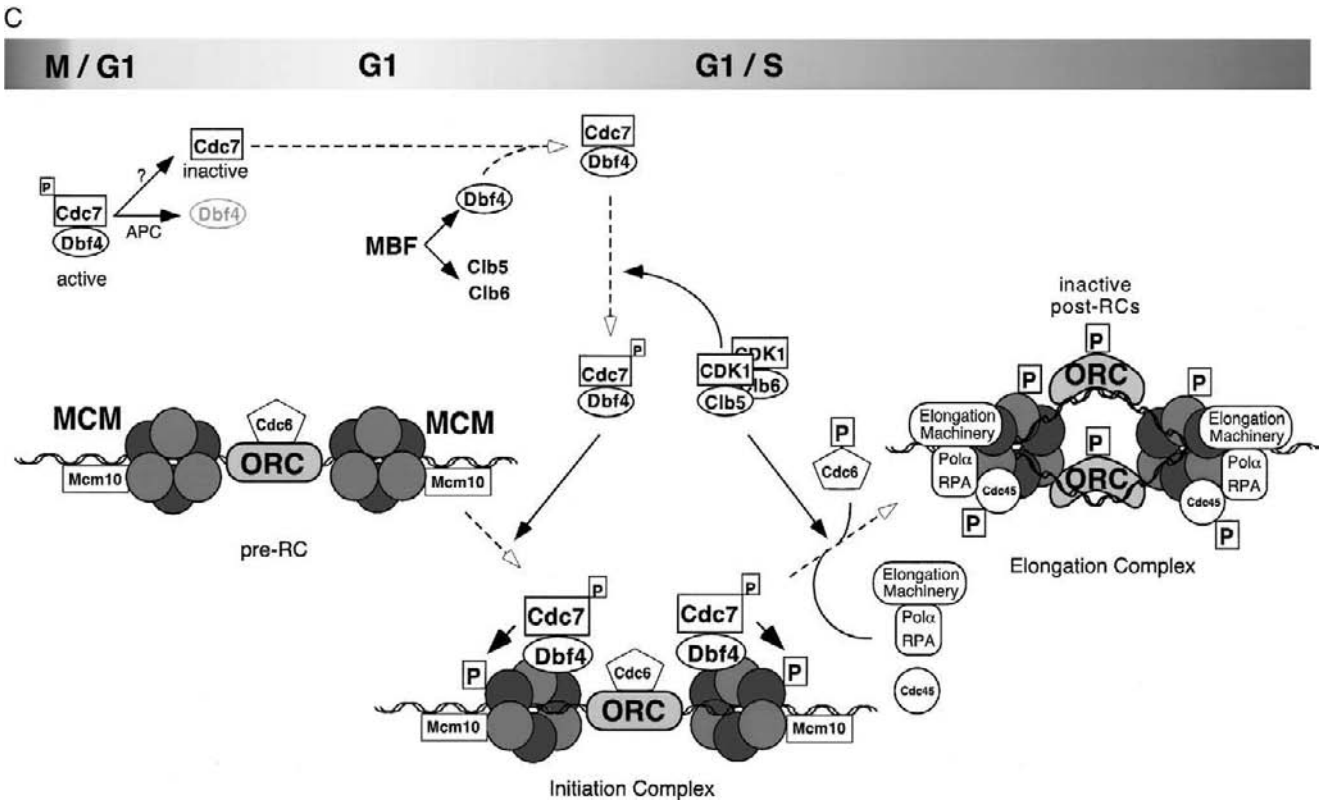


FIG. 12 *(continued)*

Jaspersen *et al.,* 1999). Thus, CDK controls the initial event leading to its own inactivation since APC/Cdc20 activation is triggered by mitotic CDK complexes to promote Clb cyclin degradation. Nevertheless, at the same time and prior to the completion of anaphase, CDK-mediated phosphorylation of the Sic1 CDK inhibitor, the Swi5 transcription factor, and the APC/Hct1 Clb2 degrader prevents its own full inactivation. Phosphorylation of the Sic1 protein induces its degradation (Schwob *et al.,* 1994; Visintin *et al.,* 1998), whereas phosphorylated Swi5 and APC/Hct1 are inactive (Moll *et al.,* 1991; Dynlacht, 1997; Jaspersen *et al.,* 1999). Once anaphase has been completed and sister chromatids have been segregated, this situation is reversed by Cdc14, the phosphatase that counteracts the CDK phosphorylation of Sic1, Swi5, and APC/Hct1 (Visintin *et al.,* 1998; Jaspersen *et al.,* 1999). Under Sic1 and Hct1/Cdh1 action, CDK is fully inactivated, and exit from mitosis or entry into G_1 are promoted irreversibly. This Sic1 and Hct1/Cdh1 mechanism is active throughout G_1 (Figs. 11 and 12A). In addition to the Cdc14-triggered action on CDKs, we recently suggested that the Cdc6 initiation protein cooperates with the Sic1 and Hct1 network in CDK inactivation late in mitosis by acting as an inhibitor of CDK1/Clb cyclins (Calzada *et al.,* 2001). Our results suggest that the amino terminus of Cdc6, through which the initiator protein interacts *in vivo* with Cdc28 and Cdc4 (Elsasser *et al.,* 1996; Drury *et al.,* 1997; Calzada *et al.,* 2000), is required for this inhibitory role and, consistent with this, *cdc6* mutants lacking this domain show defects in the exit from mitosis. By lowering the CDK-associated kinase activity, Cdc6 may cooperate in mitotic exit and may also favor its own loading on chromatin to initiate prereplicative complex formation at origins of replication.

2. Origin Recognition Complex Bound Permanently to ARSs?

ORC complexes can be understood as landing paths for other proteins or protein complexes and the replication machinery required to initiate DNA synthesis at origins. Once DNA replication has been initiated from a given origin of replication, this origin remains in the post-RC state and is thus unable to drive a new initiation event. Post-RC footprints are present at origins during S, G_2, and most of M phase (Diffley *et al.,* 1994), and they alternate with pre-RCs footprints. Consistent with the continuous presence of ORC complexes at origins, both DNA footprinting analysis (Diffley *et al.,* 1994) and immunoprecipitation analysis (Liang and Stillman, 1997) suggest that ORC binds to origin-associated chromatin immediately after the initiation event and that it remains there throughout the cell division cycle. It is unknown whether ORC proteins form an active complex immediately after binding to ARSs, but one striking possibility is that, derived from the CDK-dependent mechanism that prevents rereplication, the ORC complex could remain noncompetent for pre-RC formation (Fig. 12B). In fact, consistent with this idea, at least one ORC subunit (Orc6) is phosphorylated as soon as cells enter S phase and persists in that state up to the M to G_1 transition (Liang and Stillman, 1997). It

has been suggested that Orc6 phosphorylation depends on S-CDKs since mutation of the S-CDK putative sites avoids Orc6 phosphorylation (Liang and Stillman, 1997). Significantly, Orc6 appears to be dephosphorylated at the time of pre-RC assembly at origins of replication. This evidence supports the idea that phosphorylated Orc6 could be inactive and that in this way the assembly of pre-RCs is prevented. Orc6 dephosphorylation or *de novo* synthesis would be one of the multiple steps required at the end of mitosis to reset the ability of ORC to form pre-RC complexes. It has been shown that not only Orc6 but also Orc1 and Orc2 are *in vitro* substrates for CDK1/Clb5 (Mizushima *et al.,* 2000). However, it is not known whether this kinase, or indeed other CDKs, is able to phosphorylate these ORC subunits *in vivo* or whether these hypothetical modifications have any effect on the ARS–ORC–Cdc6 interaction, as has been suggested (Mizushima *et al.,* 2000).

3. Loading of Cdc6 Allows the Formation of pre-RCs

Cdc6 is an unstable protein that is rapidly degraded at the G_1 to S phase transition (Piatti *et al.,* 1996; Drury *et al.,* 1997; Sánchez *et al.,* 1999) and that must be synthesized *de novo* in each cell cycle. In any circumstance, when cells enter a new cell division cycle a new round of Cdc6 synthesis is required in order for pre-RCs assembly to occur. Our recent work suggests that Cdc6 also acts as a CDK1/Clb2 inhibitor at the end of mitosis, cooperating with Sic1 and APC/Hct1 in the inactivation of mitotic CDKs (Fig. 12A) (Calzada *et al.,* 2001).

The Cdc6 protein interacts with the ORC complex (Liang *et al.,* 1995; Mizushima *et al.,* 2000) in a pre-ORC–ARS assembly-dependent fashion (Mizushima *et al.,* 2000). These data suggest that the Cdc6 protein is involved in pre-RC formation in two distinct ways. First, it participates in removing the CDK block to pre-RC formation. Second, it is required for the assembly of pre-RC (Cocker *et al.,* 1996). Interestingly, Cdc6 may help its own loading on ARS–ORC complexes by inhibiting mitotic CDKs. Cdc6 has putative NTP binding domains, and although nucleotide binding has not been demonstrated, these domains are essential for Cdc6 to function as an initiator (Perkins and Diffley, 1998; Weinreich *et al.,* 1999). The A motif is essential for interaction with ORC onto origins of replication, and the B motif is essential for the subsequent loading of Mcms (Perkins and Diffley, 1998; Weinreich *et al.,* 1999).

What role does Cdc6 play in the assembly of pre-RCs? Recent *in vitro* studies suggest that the interaction between ORC and origins and between ORC and Cdc6 depends on ATP, and that a mutant Cdc6 lacking functional A or B motifs prevents the oligomerization of ORC (Mizushima *et al.,* 2000; Seki and Diffley, 2000). These data point to the possibility that Cdc6 might modify the ability of ORC to interact with replication origins. Thus, Cdc6 would cause a reconformation of the ORC structure. Additionally, it has been suggested that the initiator protein modulates the DNA-binding activity of ORC by restricting it to functional

origin sequences (Mizushima *et al.,* 2000). ATP and functional NTP-binding motifs on Cdc6 are required for this modulation, suggesting that ATPase activity may be involved in ORC final conformation in order to interact properly with ARSs *in vivo* (Mizushima *et al.,* 2000).

Cdc6 remains attached to chromatin, most likely via its interaction with ORC, throughout the G_1 phase (Weinreich *et al.,* 1999). Thus, during the G_1 interval a window exists that allows MCM complex binding to ARS–ORC–Cdc6 and the formation of pre-RCs (Piatti *et al.,* 1996). Since Cdc6 function and ATP binding are necessary for MCM complex attachment to origins of replication (Tanaka *et al.,* 1997; Donovan *et al.,* 1997; Perkins and Diffley, 1998; Weinreich *et al.,* 1999; Mizushima *et al.,* 2000), it could be argued that the Cdc6-dependent conformational change in ORC is both involved in the proper recognition of ARSs and required for the recruitment of the MCM complex to form a fully functional pre-RC.

4. Mcm10 Prior to Mcm Complex

Although the function of Mcm10 in the initiation of DNA replication is not known in detail, the actual binding of the MCM complex to the ARS–ORC–Cdc6 structure also seems to be dependent on Mcm10 binding (Homesley *et al.,* 2000). This hypothesis explains the defects associated with *MCM10* mutants regarding the initiation of DNA replication.

Mcm10 protein levels seem to remain constant along the cell cycle and the protein is located in the nucleus (Merchant *et al.,* 1997). Additionally, it has been reported that the Mcm10 protein remains associated with chromatin throughout the cell division cycle (Homesley *et al.,* 2000). Thus, the role of Mcm10 in pre-RC assembly remains to be elucidated.

5. The Mcm Complex

As mentioned previously, binding of the MCM complex to chromatin is only possible if Cdc6 is actually bound to ARS–ORC complexes (Fig. 12B) (Aparicio *et al.,* 1997; Tanaka *et al.,* 1997; Mizushima *et al.,* 2000; Seki and Diffley, 2000). Thus, not only the ability to associate with chromatin but also the timing of this event depends on Cdc6. Significantly, the binding of the MCM complex to chromatin also depends on other factors since even in the presence of Cdc6 at origins at least Mcm7 does not interact with ARSs (Tanaka *et al.,* 1997).

MCM associates with chromatin in G_1, gradually disassociates during S phase, and remains unassociated in G_2 and mitosis (Aparicio *et al.,* 1997; Donovan *et al.,* 1997; Tanaka *et al.,* 1997). Supporting previous observations (Hennessy *et al.,* 1990; Yan *et al.,* 1993; Dalton and Whitbread, 1995), it has recently been reported that the subcellular distribution of some Mcm proteins is cell cycle regulated (Labib *et al.,* 1999; Nguyen *et al.,* 2000).

As argued previously, the CDK-regulated subcellular localization of Mcm proteins or the CDK-regulated Mcm binding to chromatin could be an important part of the CDK-mediated control over rereplication (Labib *et al.,* 1999; Nguyen *et al.,* 2000; Tye, 1999) since the decrease in CDK1/Clb levels at the end of mitosis seems to be required for rapid entry of Mcm proteins into the nucleus (Nguyen *et al.,* 2000). In G_2 and mitosis, CDK1/Clb kinases maintain Mcm proteins in the cytoplasm by promoting their nuclear export. At the end of mitosis, when CDK1/Clb activity decreases, Mcm proteins reenter the nucleus to bind ARS–ORC–Cdc6 complexes and remain in that state up to the initiation of DNA replication, when CDK1/Clb5,6 activities increase (Nguyen *et al.,* 2000). Excess nuclear Mcm proteins will be exported to the cytoplasm by CDK1/Cln complexes once cells have passed through START (Labib *et al.,* 1999) or as a consequence of the increase in CDK1/Clb activity even in the absence of S phase initiation (Labib *et al.,* 1999; Nguyen *et al.,* 2000). It is possible that Mcms released from chromatin, as a consequence of Cdc6 degradation, can be exported from the nucleus in G_1-arrested cells in order to prevent reformation of pre-RCs under pheromone treatment (Labib *et al.,* 1999).

B. Activation of Competent Origin after the Formation of Pre-RCs

Although a given origin with an assembled pre-RC is competent for replication, DNA synthesis only initiates from it when S phase CDKs and DDK are activated at the end of G_1. Kinase activation is the point of transition from G_1 to S phase and induces the initiation of DNA synthesis, the disassembly of pre-RCs at origins once replication has been fired from them, and as a direct consequence of the disassembly process, it promotes a block to new rounds of DNA replication that lasts up to the end of mitosis (Fig. 12C).

1. Cdc7/Dbf4 on Each Origin of Replication

Cdc7/Dbf4 (DDK) is a serine/threonine protein kinase required for the onset of DNA synthesis. Cdc7, the catalytic subunit, is present at constant levels throughout the cell division cycle (Dixon and Campbell, 1997; Johnston *et al.,* 1999), although its phosphorylation pattern changes (Dixon and Campbell, 1997; Johnston *et al.,* 1999): Hypophosphorylated in G_1, the protein becomes phosphorylated, and thus activated, as soon as cells enter S phase (Yoon *et al.,* 1993). Dbf4, the regulatory subunit, oscillates and is present in cycling cells from G_1 to the end of mitosis when it is actively degraded by an APC-dependent mechanism (Cheng *et al.,* 1999; Jackson *et al.,* 1993; Kitada *et al.,* 1993; Yoon *et al.,* 1993; Ferreira *et al.,* 2000). In this context, the disappearance of DDK activity at the M to G_1 boundary ensures that newly formed pre-RCs do not fire prematurely. DDK activity peaks at

the G_1 to S phase transition and depends on S-CDK activation (Nougarede *et al.,* 2000) to jointly trigger the initiation of DNA replication (Fig. 12C).

It has been shown that DDK interacts with ARSs (Dowell *et al.,* 1994). In fact, strong genetic and biochemical evidence supports the notion that DDK interacts with MCM proteins. Although mutation of *MCM5* bypasses the requirement for Cdc7/Dbf4 (Hardy *et al.,* 1997), the Mcm5 protein does not appear to be a substrate for DDK, at least *in vitro* (Lei *et al.,* 1997). This suggests that another Mcm protein could be the transducer of Cdc7/Dbf4 regulation over initiation of DNA replication. Mcm2 interacts with DDK, as shown by two-hybrid analysis, and this Mcm protein is also a good *in vitro* substrate for the kinase (Lei *et al.,* 1997). These data suggest that the essential function of DDK in DNA replication is exerted on the MCM complex and that Mcm2 may be the only essential target of regulation by Cdc7/Dbf4. This regulation may involve both direct interaction and phosphorylation of the Mcm2 protein, although *in vitro* data also support the possibility that Mcm3, Mcm4, and Mcm6 might be substrates of DDK (Lei *et al.,* 1997).

A simple assumption (Fig. 12C) that explains the role and interactions of these components at the G_1 to S phase transition is that Cdc7 is inactive through G_1 due to both its hypophosphorylated state and the lack of the regulatory subunit Dbf4. At the end of G_1, Dbf4 is synthesized, and after binding to Cdc7 it activates the DDK-associated kinase, although fully functional DDK requires prior activation of S-CDKs (Nougarede *et al.,* 2000). Dbf4 targets DDK to origins of replication (Dowell *et al.,* 1994); there, the kinase interacts with the MCM complex and, presumably by phosphorylating one (Mcm2) or more components of this complex, may induce a conformational change that leads to MCM activation. Following this interpretation, the bypass of Cdc7/Dbf4 function observed in *MCM5* mutants can be explained if such mutants are able to transform the MCM complex structure into a constitutively active complex dependent only on S-CDKs activation.

2. Cdk1/S Phase Cyclins

Initiation of DNA replication can only occur when DDK and S-CDKs are active. The dual role of S-CDKs activity in the regulation of DNA synthesis, essential for the initiation event and also required to block rereplication, could explain why two different kinase complexes (S-CDKs and DDK) participate in origins of replication firing.

Two distinct B-type cyclins, Clb5 and Clb6, are involved in CDK1 activation at the G_1/S transition, and although they may have overlapping roles, it is likely that they perform different functions in the activation of DNA synthesis. By this means, activation of origins of replication is unaffected by the absence of the *CLB6* gene, but *CLB5* deletion mutants fail to fire late origins and the entire genome is replicated by forks from early origins (Donaldson *et al.,* 1998). This suggests that CDK1/Clb5 and CDK1/Clb6 can fire early origins, but only CDK1/Clb5 can

fire late origins. The meaning of this distinction in terms of S phase regulation is unknown but this could well be the result of different substrate specificity or due to differences in the expression pattern of both cyclins. Alternatively, it could be due to differences in the S-CDK levels required to fire early and late origins (Diffley, 1998). Since the pattern of expression does not differ significantly (Epstein and Cross, 1992; Richardson *et al.,* 1992; Schwob and Nasmyth, 1993; Dirick *et al.,* 1995; Stuart and Wittenberg, 1995) and because the associated kinase activity seems to be similar (Oehlen *et al.,* 1998), we favor the first possibility.

It is thought that the dual role of S-CDKs in DNA replication is based on the phosphorylation of key substrates of pre-RC complexes, and that these phosphorylation processes drive both the initiation event and the conformational changes that preclude an eventual reinitiation of genome replication (Dutta and Bell, 1997; Tye, 1999; Lee and Bell, 2000). Although all experimental evidence supports this model, direct biochemical data to fully confirm it are still lacking. In principle, any of the components of pre-RCs would be good candidates as substrates of CDK1/Clb5 and Clb6. Phosphorylation of chromatin-bound Cdc6 could be a signal for the irreversible removal of Cdc6 and for activation of downstream events, such as the formation of the initiation complex (Fig. 12C) (Lee and Bell, 2000). This reaction is irreversible since CDK-phosphorylated Cdc6 is targeted for rapid proteolysis (Drury *et al.,* 1997) after ubiquitination (Sánchez *et al.,* 1999). It has consistently been shown that Cdc6 is a substrate for both CDK1/Cln and CDK1/Clb complexes (Elsasser *et al.,* 1996; Calzada *et al.,* 2000; Drury *et al.,* 2000). DDK could also be a substrate of S-CDKs and its phosphorylation might be important for its regulation (Yoon *et al.,* 1993; Johnston *et al.,* 1999).

Another possible target of CDK1-Clb is the Cdc45 protein. Cdc45 binds replication origins during G_1 (Aparicio *et al.,* 1997). This binding or association with chromatin becomes stronger as the cell progresses into late G_1 (Zou and Stillman, 1998). The tight chromatin association is delayed in cells deleted for *CLB5* and *CLB6,* suggesting that CDK1-dependent Clb5 and -6 activities are required for a timely regulated, tight binding of Cdc45 to chromatin (Zou and Stillman, 1998). Cdc45 is found tightly associated with chromatin in cells deprived of Cdc7 near the G_1/S boundary (Zou and Stillman, 1998), suggesting that the binding of Cdc45 to origins of replication is independent of Cdc7/Dbf4 function.

Members of the ORC complex are also potential *in vivo* substrates of CDKs and research efforts have been conducted to address this point (Liang and Stillman, 1997). It is known that changes in the electrophoretic mobility of Orc6 can be attributed to phosphorylation, and that these changes as well as Orc2 phosphorylation could be involved in some critical conformational changes in ORC along the cell division cycle (Liang and Stillman, 1997). Recently, it was proposed that changes in the conformation of ORC and ORC-dependent ATPase activity are linked to pre-RCs disassembly and the actual inactivation of the ORC (Lee and Bell, 2000). It has been suggested that these conformational changes are dependent on ssDNA–ORC interaction since ssDNA appears in the transition from dsDNA

to ssDNA at origins of replication during the DNA unwinding step (Lee and Bell, 2000). Although CDK-mediated phosphorylation might be involved in these processes, this aspect has not been addressed. Thus, the global and coordinated action of CDK and DDK on different components of prereplicative complexes will initiate DNA synthesis.

It is thought that CDKs are involved not only in the phosphorylation of initiators but also in the regulation of replication factors involved in the elongation step of DNA synthesis. It is known that human CDKs phosphorylate one of the three subunits of the ssDNA binding protein RPA and two of four subunits of DNA polymerase α-primase, both remaining phosphorylated from the onset of S phase until the end of mitosis (Nasheuer *et al.,* 1991; Dutta and Stillman, 1992). In the yeast *S. cerevisiae,* three genes (*RFA1, RFA2,* and *RFA3*) encode the subunits of the protein complex with RPA activity. All RFA genes are required for cell viability. These and other results clearly indicate that in budding yeast RPA activity is essential for DNA replication initiation (Foiani *et al.,* 1997; Wold, 1997). In this model system, it has been suggested that RPA might mediate the action on origins of replication of S phase CDKs and DDK (Tanaka and Nasmyth, 1998; Zou and Stillman, 2000). Nevertheless, RPA association with origins depends on S-CDKs and DDK activities; also, ARSs recruitment of DNA polymerase α-primase relies on RPA activity (Tanaka and Nasmyth, 1998). Significantly, the timing of RPA association with early origins of replication differs from that for late origins, a process regulated by the kinase Rad53 (Tanaka and Nasmyth, 1998). Moreover, RPA, Cdc45, and Mcm2 associate with one another at the onset of S phase (Zou and Stillman, 2000), although there are also differences in the timing of association of Cdc45 and DNA polymerase α-primase with early and late origins of replication (Aparicio *et al.,* 1999). These findings suggest that the formation of a complex containing Mcm proteins, Cdc45, RPA, and DNA polymerase α-primase at each individual origin probably depends on Rad53 for appropriate timing of association. Probably, the previously mentioned proteins participate in DNA unwinding at origins, thus forming the structure of the initiation replication complex needed for initiation and for the recruitment of other proteins to form replication elongation complexes. The development of an *in vitro* assay would be appropriate in order to understand how the sequence of events occurs in the initiation of DNA replication. Figure 12C helps to visualize current knowledge of the sequence of events coordinated by S-CDKs at the initiation of S phase.

C. Connection with the Replication Machinery?

1. Are Mcm Complexes Eukaryotic Helicases?

Eukaryotic Mcms interact with each other to form different complexes, including Mcm2–7, Mcm4–Mcm6–Mcm7, Mcm2–Mcm4–Mcm6–Mcm7, or Mcm3–Mcm5 (Ishimi, 1997; Thommes *et al.,* 1997; Sherman and Forsburg, 1998). Biochemical

studies have shown that only a dimeric complex formed by two Mcm4–Mcm6–Mcm7 heterotrimers is capable of sustaining DNA helicase, ssDNA binding, and DNA-dependent ATPase activities (Ishimi, 1997; You *et al.,* 1999; Lee and Hurwitz, 2000). It has also been shown that interaction of Mcm2 or Mcm3–Mcm5 with the heterotrimer Mcm4–Mcm6–Mcm7 inhibits helicase activity (Ishimi *et al.,* 1998; Lee and Hurwitz, 2000), suggesting that the trimeric form might be the catalytic core of the Mcm complex and that Mcm2 or Mcm3–Mcm5 might regulate the activity of this complex.

Mcm protein localization (Mcm4 and Mcm7) changes from origins of replication to interorigin regions during S phase in *S. cerevisiae* (Aparicio *et al.,* 1997), and Mcm proteins are also required for these changes in localization as well as for the progression of the replication fork (Aparicio *et al.,* 1997; Labib *et al.,* 1999, 2000). These observations are consistent with a role for the MCM complex as DNA helicases (Baker and Bell, 1998; Leatherwood, 1998; Tye and Sawyer, 2000). Nevertheless, the *S. pombe* Mcm4–Mcm6–Mcm7 complex shows very limited helicase activity *in vitro* and is only capable of unwinding very short duplex DNA fragments (Ishimi, 1997; Lee and Hurwitz, 2000). Owing to the limited processivity of the fission yeast Mcm4–Mcm6–Mcm7 complex, it was suggested that additional factors are required *in vivo* to fulfill the helicase activity role (Ishimi, 1997; Lee and Hurwitz, 2000). Analysis with forked DNA structures revealed that the presence of single-stranded tails stimulates the processive helicase activity of the Mcm4–Mcm6–Mcm7 trimeric form (Lee and Hurwitz, 2000). Evidence obtained in *S. cerevisiae* and *S. pombe* supports the notion that MCM complexes are eukaryotic helicases, although this hypothesis needs further confirmation in multicellular systems.

2. Cdc45 also Goes with the Replication Fork

Genetic studies suggest a role for Cdc45 in the activation of MCM complexes. Among the most significant lines of evidence is that the loss of Cdc45 function can be bypassed by MCM mutants (Hennessy *et al.,* 1991). *In vitro,* RPA can stimulate MCM-associated helicase activity (Herendeen and Kelly, 1996), suggesting that it might also be involved in the activation of the helicase *in vivo* in yeast. Therefore, it is possible that Cdc45 and RPA collaborate to activate or to increase the processivity of the MCM helicase complex (Zou and Stillman, 2000). Consistent with a participative role in origin of replication unwinding, Cdc45 and RPA are required for DNA polymerase α loading onto chromatin (Tanaka and Nasmyth, 1998). The function of Cdc45 is required throughout S phase because Cdc45 associates with MCM complexes, RPA, and polymerase α during S phase (Zou and Stillman, 2000) and moves from origin to interorigin DNA regions as S phase progresses (Aparicio *et al.,* 1997; Tanaka and Nasmyth, 1998). These findings support the idea that Cdc45, RPA, and MCM complexes progress with replication forks and that Cdc45, together with RPA, may continue to stimulate MCM helicase activity during the elongation step of DNA replication.

V. Ploidy Maintenance in Uni- and Multicellular Eukaryotes

Once S phase has started, a new round of DNA synthesis is prevented until mitosis has been completed and daughter cells reenter a new cell division cycle. What is the molecular nature of the control mechanism ensuring a complete and single round of DNA synthesis in each cell cycle? Critical experiments carried out in yeast, both *S. cerevisiae* and *S. pombe* and in metazoans, *Drosophila,* and *Xenopus* have provided strong evidence supporting a role for CDKs in preventing rereplication once DNA synthesis has been initiated and throughout S phase, G_2, and mitosis. Thus, CDKs integrate both positive signaling to initiate replication (see Sections III.C.1 and IV.B.2) and an overall negative control, preventing extra initiation events. This control mechanism appears to be conserved from yeast to vertebrates.

In particular, extra rounds of DNA replication within the same cell division cycle are observed in *S. pombe* when *cdc13* (the major mitotic cyclin) is deleted, with some ts mutant alleles of *cdc2* (the fission yeast CDK catalytic subunit), and when the *rum1*$^+$ CKI is overexpressed (Broek *et al.,* 1991; Hayles *et al.,* 1994; Moreno and Nurse, 1994). Further demonstration of the CDK-mediated prevention of rereplication derives from experiments in *S. cerevisiae,* in which a decrease in CDK activity induces an illegal genome replication event (in the absence of cell division) in cells blocked in mitosis (Dahmann *et al.,* 1995). Also consistent with this CDK-mediated control, a pulse at the restrictive temperature of certain *cdc28* alleles promotes and additional round of DNA synthesis (A. Calzada and A. Bueno, unpublished observations). Similar evidence has been found in *Drosophila* since depletion of mitotic cyclins during embryogenesis causes cells to polyploidize (Lehner and O'Farrell, 1990; Sauer *et al.,* 1995). In *Xenopus,* the presence of CDK activity prevents the licensing of chromatin, thus blocking DNA rereplication (Hua *et al.,* 1997; Mahbubani *et al.,* 1997). Human cells have a similar control over rereplication since some conditional *CDC2* mutants undergo an extra round of DNA replication (Itzhaki *et al.,* 1997). Despite evidence suggesting that rereplication is prevented in a CDK-dependent manner in all eukaryotes, some differences exist, particularly in the details of this general control mechanism.

A. *Saccharomyces cerevisiae* and *S. pombe:* Diverse Mechanisms Controlled by CDKs

1. *Saccharomyces cerevisiae:* An Overall CDK-Mediated Control

In budding yeast, mitotic Clbs (Clb1–4) and S phase Clbs (Clb5–6) are able to block rereplication. Thus, this inhibitory mechanism covers all cell cycle stages from S phase, in which Clb-associated CDK activity starts to increase to the end of mitosis, up to the inactivation of CDK1/Clb kinases.

How do CDK1/Clbs block rereplication in *S. cerevisiae?* The mechanism by which budding yeast ensures a single round of DNA synthesis involves the prevention of origin refiring within the same cell division cycle. Assembly of prereplicative complexes at origins of replication is inhibited by CDK1/Clbs kinase activity (Dahmann *et al.,* 1995; Detweiler and Li, 1998; Calzada *et al.,* 2000). This assembly requires Cdc6 (see Section IV.A.2), an unstable protein only present in the cell from the end of mitosis to the G_1/S phase transition (Piatti *et al.,* 1995; Santocanale and Diffley, 1996; Cocker *et al.,* 1996; Detweiler and Li, 1997), and the presence of many components of pre-RC complexes that must be dephosphorylated in order to be competent for association with ARSs–ORC–Cdc6 complexes (Tye, 1999). In the following sections the sequence of events prevented by CDK1/Clbs that block pre-RCs formation is summarized.

a. CDK-Mediated Inhibition of Pre-RCs Assembly

i. Inhibition of the Transcription of Initiation Genes As explained in Section II.D, some genes involved in DNA replication initiation are transcribed during telophase (Mendenhall and Hodge, 1998). This is the case for *CDC6* and other genes involved in DNA replication (McInerny *et al.,* 1997). In particular, transcription of *CDC6* at the M/G_1 transition is regulated by Mcm1 and Swi5 (Zwerschke *et al.,* 1994; Piatti *et al.,* 1995; Aerne *et al.,* 1998) once the latter transcription factor has been imported to the nucleus at the end of mitosis (Nasmyth *et al.,* 1990; Moll *et al.,* 1991). Nuclear import of the Swi5 transcription factors is a CDK cell cycle-regulated process. The Swi5 protein is cytoplasmic up to the end of mitosis, when it becomes rapidly imported into the nucleus. The nuclear import of this transcription factor is prevented by CDK1/Clb activity (Moll *et al.,* 1991), as deduced essentially from the analysis of Swi5 mutants lacking putative CDK1 phosphorylation sites. This CDK1/Clb-mediated block of Swi5 nuclear import prevents expression of the *SIC1*-encoded CDK inhibitor and the *CDC6* gene (Schwob *et al.,* 1994; Piatti *et al.,* 1995), thus preventing the synthesis of the essential factor for the assembly of pre-RCs at origins (Cocker *et al.,* 1996). This CDK1/Clb inhibition of Swi5-dependent transcription is reversed by the action of the protein phosphatase encoded by *CDC14* (Visintin *et al.,* 1998; Jaspersen *et al.,* 1999).

ii. Phosphorylation of Initiators Preventing Pre-RC Assembly Subcellular localization of Mcm proteins may contribute to restricting pre-RC assembly to the G_1 phase of the cell cycle, particularly because Mcm localization (Hennessy *et al.,* 1990; Yan *et al.,* 1993; Whitebread and Dalton, 1995) appears to be regulated by CDK-associated kinase activity (Labib *et al.,* 1999; Nguyen *et al.,* 2000). Mcm proteins are nuclear in G_1 and are excluded from the nucleus as S phase progresses by CDK1/Clb (Labib *et al.,* 1999; Nguyen *et al.,* 2000). CDK1/Cln also promotes Mcm export from G_1 nuclei (Labib *et al.,* 1999). This interesting observation suggests that G_1 cyclins may reduce the capacity for pre-RC complexes to be assembled before DNA synthesis has been triggered from origins of replication by CDK1/Clbs during S phase. The fact that nucleus export is regulated

by B-type cyclin-associated CDK activity may help one to understand why in the G_2 phase, even in the presence of chromatin-bound Cdc6, binding of MCM complex to origins of replication remains elusive. Nonetheless, constitutive nuclear Mcm localization does not cause rereplication, even when combined with Cdc6 stabilization, suggesting that an alternative mechanism or additional components of the same mechanism are involved in the prevention of rereplication (Nguyen *et al.,* 2000). In this context, *in vitro* analysis of the assembly of initiators at origins revealed that ORC–ARS complexes are unable to load Cdc6 and/or Mcm2–7 proteins when purified from cells arrested in mitosis (Seki and Diffley, 2000). The current review suggests that ORC could also be a substrate of the inhibitory mechanism preventing reassembly of pre-RCs at origins before cells have finished mitosis. In this sense, it is appropriate to mention that Orc6 is phosphorylated from S phase to the end of mitosis, hence, it is phosphorylated in a cell division cycle-dependent manner (Liang and Stillman, 1997). In fact, it is possible that Orc6 could be a CDK1/Clb substrate since the phosphoacceptor amino acids present in this protein fulfill the CDK1 consensus requirements (Liang and Stillman, 1997). Nevertheless, it is unknown whether the combination of stable Cdc6, constitutive nuclear Mcms, and nonphosphorylatable Orc components might promote the assembly of pre-RCs at origins even in the presence of CDK kinase activity. Perhaps other components, such as Mcm10, are involved in this CDK-controlled multiple mechanism preventing rereplication.

iii. Targeted Proteolysis of Initiators Although during the past decade there was considerable interest in the hypothesis that CDK-induced targeted proteolysis of key elements of pre-RCs could be the mechanism by which eukaryotes prevented rereplication (Nishitani and Nurse, 1995), this hypothesis failed to fully explain all the experimental observations made in both unicellular and multicellular eukaryotes. Nonetheless, targeted proteolysis is an important part of such a mechanism, at least in budding yeast. In particular, it is known that the degradation of Cdc6 depends on SCF/Cdc4 and that it is triggered by CDK-mediated phosphorylation of the initiator protein (Drury *et al.,* 1997; Elsasser *et al.,* 1999; Sánchez *et al.,* 1999; Calzada *et al.,* 2000; Drury *et al.,* 2000). Since pre-RC formation depends strictly on Cdc6 (Cocker *et al.,* 1996; Santocanale and Diffley, 1996; Liang and Stillman, 1997), Cdc6 instability is sufficient to efficiently prevent pre-RC assembly at origins. Nevertheless, as mentioned previously, Cdc6 proteolysis is only one part of a multiple CDK-controlled mechanism of ploidy maintenance in the budding yeast *S. cerevisiae* (Nguyen *et al.,* 2001). Current knowledge of CDK control over ploidy maintenance and substrates is summarized in Fig. 13.

2. *Schizosaccharomyces pombe:* Is the Emphasis on CDK-Targeted Proteolysis?

As in the budding yeast *S. cerevisiae,* the activity of CDKs may govern the prevention mechanism that blocks rereplication in *S. pombe* cells. Evidence suggesting

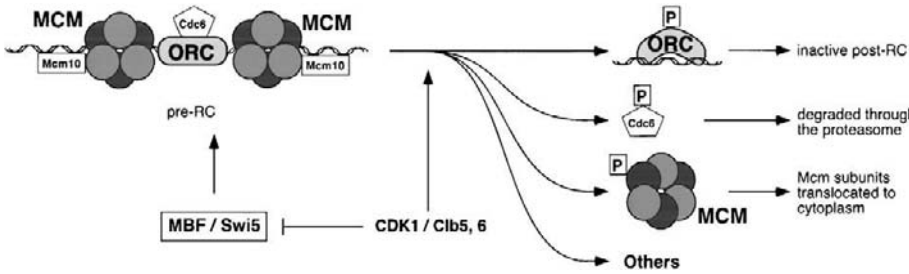


FIG. 13 Schematic view of the major molecular events regulated by CDK1/Clb5,6 in the prevention of rereplication in *S. cerevisiae*.

such a CDK-controlled mechanism is that cells deleted for *cdc13,* an essential mitotic B-type cyclin, undergo successive rounds of DNA replication in the absence of cell division (Hayles *et al.,* 1994). Furthermore, some *cdc2* ts mutants undergo an extra S phase when the Cdc2 protein has been degraded (Broek *et al.,* 1991). Also consistent with this proposed role for CDK, overproduction of the CKI Rum1 protein results in a continuous DNA synthesis phenotype comparable to the one observed in Δ*cdc13 S. pombe* cells. It is appropriate to mention here that the Rum1 protein is an effective *in vivo* inhibitor of CDK/Cdc13 complex activity (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). Possible candidate targets of this CDK/Cdc13-mediated prevention mechanism include all proteins that play a role in the initiation of DNA replication, such as the initiators presumably forming the prereplicative complexes in fission yeast: Cdc18, members of the ORC and MCM families, and Cdt1.

That CDK might prevent rereplication by regulating the proteolysis of Cdc18 was first suggested by the repeated rounds of DNA replication observed when the (Cdc18) initiator protein was overexpressed (Nishitani and Nurse, 1995). Consistent with this notion, a decrease in CDK activity induced by the overexpression of Rum1 leads to the accumulation of Cdc18 (Jallepalli and Kelly, 1996). The Cdc18 protein contains six CDK consensus phosphorylation sites. A protein with the suitable residues mutated to alanines is stable relative to the wild-type protein (Jallepalli *et al.,* 1997; López-Girona *et al.,* 1998); in fact, the initiator protein is targeted for proteolysis by CDK direct phosphorylation after ubiquitination by Pop1 and Sud1, which are homologs of the Cdc4 protein of *S. cerevisiae* (Kominami and Toda, 1997; Jallepalli *et al.,* 1998; Kominami *et al.,* 1998). These observations led to the suggestion that CDK-controlled proteolysis of Cdc18 might ensure single rounds of DNA replication during each cell cycle in fission yeast. Nevertheless, it is not likely that a single-substrate mechanism ensures ploidy maintenance in *S. pombe* since it has been shown that other pre-RC components are modified in a CDK-dependent manner to prevent the reformation of prereplicative complexes before cells exit from mitosis. It is true that *pop1* and *sud1* mutants polyploidize

and that these mutants accumulate the Cdc18 protein, but extra rounds of DNA replication in these circumstances are likely the consequence of the accumulation of the CDK inhibitor Rum1 (Kominami and Toda, 1997; Jallepalli *et al.,* 1998).

ORC components are CDK substrate candidates involved in the control mechanism preventing rereplication. There is no evidence for the relocalization of SpORC subunits to the cytoplasm at any point during the different stages of the cell division cycle. In fact, SpORC subunits cofractionate with chromatin, even from cells arrested at mitosis (Lygerou and Nurse, 1999). However, at least one ORC subunit, SpOrc2, is modified in a cell cycle-dependent fashion. SpOrc2 is phosphorylated in mitosis, when CDK/Cdc13 activity is high, most likely in a CDK-dependent manner since SpOrc2 and the CDK catalytic subunit interact, at least in a two-hybrid assay (Leatherwood *et al.,* 1996). Transition to the G_1 phase is followed by the appearance of the faster migrating form of SpOrc2 and the accumulation of Cdc18 (Lygerou and Nurse, 1999). These observations support the hypothesis that phosphorylation of SpOrc2 may change its capacity to interact with other proteins to form pre-RC complexes, thus suggesting a role for this CDK-controlled step in the control ensuring ploidy maintenance in this organism.

All six MCM protein components are constitutively located at the nucleus throughout the cell division cycle (Maiorano *et al.,* 1996; Okishio *et al.,* 1996; Sherman and Forsburg, 1998). Inside the nucleus, all six proteins remain assembled to each other to form full MCM complexes (Pasion and Forsburg, 1999). Nevertheless, *S. pombe* MCM complexes are bound to chromatin only during G_1 since they disassociate from it as S phase progresses and remain in that state until the end of mitosis. This indicates that MCM assembly to chromatin is somehow regulated in a cell cycle-dependent manner. Whether chromatin binding is regulated by direct phosphorylation by CDKs or DDK, as proposed in budding yeast (see Section V.A), or whether it is dependent on the presence of the Cdc18 initiator protein or perhaps regulated by another unknown mechanism remain to be elucidated.

Finally, Cdt1 has recently been identified as a component required for chromatin loading of MCM complexes in *S. pombe* (Nishitani *et al.,* 2000). The periodic expression of the *cdt1*$^+$ gene is regulated by the transcriptional machinery of G_1 (Hofmann and Beach, 1994), suggesting that if Cdt1 protein levels also fluctuate it may represent another level of control involved in the mechanism preventing rereplication in the fission yeast *S. pombe*. Again, the Cdt1 protein periodicity, its control, and its relationship with periodic MCM assembly to chromatin remain to be elucidated.

B. Multicellular Eukaryotes

Increasing evidence suggests that cell cycle control over DNA rereplication in multicellular systems also involves the inhibition of pre-RC formation once

S phase has been initiated up to the end of mitosis. In 1988, Blow and Laskey suggested that DNA replication was regulated by the nuclear localization of a "licensing factor," essential to render chromatin competent for replication. This licensing factor would be inactivated (excluded from the nucleus) during S phase progression and would only reaccumulate in the nucleus at the end of mitosis, once the nuclear membrane had been broken down (Blow and Laskey, 1988). Candidates for forming part of the licensing factor are components of pre-RCs, such as Cdc6 and Mcm proteins, although changes in the nuclear/cytoplasmic localization of CDKs also account for the control of replication and, therefore, play the role of the licensing factor (Hua *et al.,* 1997). A biochemical approach in *Xenopus* allowed the identification of the so-called replication licensing factor (RLF) and, in particular, it has been suggested that the RLF-B subfraction would be a good candidate for the maintenance of ploidy in the frog (Tada *et al.,* 1999).

Unlike in yeast, in metazoans the subcellular localization of Mcm proteins does not change during the cell division cycle and is exclusively nuclear (Fujita, 1999). In *Xenopus,* CDK activity negatively regulates the XlMcm protein–chromatin interaction. In fact, CDK phosphorylates XlMcm4 and treatment of S phase chromatin with exogenously added M phase CDK dissociates XlMcms from DNA (Hendrickson *et al.,* 1996), suggesting that *in vivo* M phase CDK may suppress reloading of free nuclearly located XlMcms onto chromatin. A similar understanding of how cells block rereplication derives from studies in human cells, in which CDK/cyclin B complexes phosphorylate HsMcm2 and HsMcm4 both *in vitro* and *in vivo*. CDK inactivation leads to the progressive disappearance of these HsMcm2 and -4 phosphorylated forms, correlating with increasing levels of chromatin-bound HsMcms (Fujita *et al.,* 1998). Also in contrast to the regulation of the initiator protein in yeast, human Cdc6 (HsCdc6) protein levels do not fluctuate during the cell division cycle (Saha *et al.,* 1998), although the protein is actively destroyed in an APC-dependent manner in quiescent cells (Petersen *et al.,* 2000). Comparable to HsMcm proteins, HsCdc6 binding to chromatin is regulated by control of its subcellular localization (Williams *et al.,* 1997; Liang and Stillman, 1997; Saha *et al.,* 1998; Petersen *et al.,* 1999). HsCdc6 is nuclear during the G_1 phase but translocates to the cytoplasm at the G_1/S phase boundary (Liang and Stillman, 1997; Petersen *et al.,* 1999). Several lines of evidence suggest that the nuclear import/export of HsCdc6 is regulated by CDK phosphorylation. First, a mutant HsCdc6 lacking CDK consensus phosphorylation sites is located in the nucleus constitutively. Furthermore, ectopic expression of cyclin A promotes the exclusion of the initiator protein from the nucleus (Petersen *et al.,* 2000). In *Xenopus,* XlCdc6 is not rapidly degraded at the initiation of S phase, as happens in yeast. Although XlCdc6 protein levels remain constant during the cell division cycle, the situation differs from humans in that XlCdc6 always remains nuclear but appears to move from the chromatin to the nuclear membrane (Coleman *et al.,* 1996). All these data support the hypothesis that in metazoans, like the control mechanism in yeast, rereplication is blocked by

inhibition of the reassembly of pre-RCs at origins. This prevention mechanism is most likely based on the incapacity of Cdc6, Mcms, and Orc proteins to interact with each other and with origins of replication when they are phosphorylated by CDKs.

The mechanism limiting the initiation of DNA synthesis events in metazoans might also involve Orc proteins. In both *Xenopus* and mammals, ORC components are displaced from chromatin during mitosis, reassociating with DNA during the G_1 phase (Coleman *et al.,* 1996; Romanowski *et al.,* 1996b; Hua *et al.,* 1997; Abdurashidova *et al.,* 1998). Expression of Orc2–5 proteins is not cell cycle regulated (Quintana *et al.,* 1997, 1998; Saha *et al.,* 1998), and in fact *ORC2–5* mRNA is detected in nonproliferating tissues (Quintana *et al.,* 1998). However, when chromatin-bound ORC complexes are purified from mitotic cells they do not contain all Orc protein subunits, suggesting that some Orc subunits disassociate from ORC hexamers (Fujita, 1999). Findings in other organisms support this conclusion. In *Xenopus,* biochemical analyses indicate that XlOrc2 is excluded from chromatin and also from the nuclear matrix during mitosis (Coleman *et al.,* 1996). Nevertheless, in *Drosophila,* DmOrc2 remains bound to chromosomal DNA in mitosis (Pak *et al.,* 1997), although DmOrc1 protein levels change during the cell division cycle and during development, accumulating in proliferating cells late in G_1 and S phase (Asano and Wharton, 1999). Furthermore, ectopic expression of DmOrc1 results in a general DNA replication in cells in which replication is normally restricted (Asano and Wharton, 1999), suggesting that DmOrc1 may be involved in limiting the DNA replication mechanism in flies. In hamster cell lines in culture, it has been shown that ORC components are involved in the rereplication prevention mechanism. Hamster Orc1 and Orc2 are constitutively located in the nucleus throughout the cell cycle, but only Orc2 is stably bound to chromatin. Biochemical approaches suggest that hamster Orc1 is bound to chromatin as cells progress through the G_1 phase but is not associated with chromatin during mitosis and early G_1 (Natale *et al.,* 2000).

Cell fusion experiments initiated by Guttes and Guttes (1969) and Rao and Johnson (1970) suggested the existence of a prevention mechanism that avoids rereplication after S phase initiation. The experimental data summarized here suggest that this mechanism is probably based on the inhibition of prereplicative complex reassembly at origins of replication once DNA synthesis has been initiated from them. Although this block to reassembly is probably mediated by the phosphorylating activity of CDK complexes, this hypothesis does not exclude the existence of additional replication inhibitors. Geminin, a protein described in *Xenopus,* may be one of them. Geminin accumulates from S phase to mitosis, inhibiting the loading of Mcm proteins onto chromatin (McGarry and Kirschner, 1998). This protein acts through the inhibition of Cdt1, a replication factor required for Mcm loading at origins (Wohlschlegel *et al.,* 2000). These properties match the expected characteristics for a rereplication inhibitor. Nevertheless, the

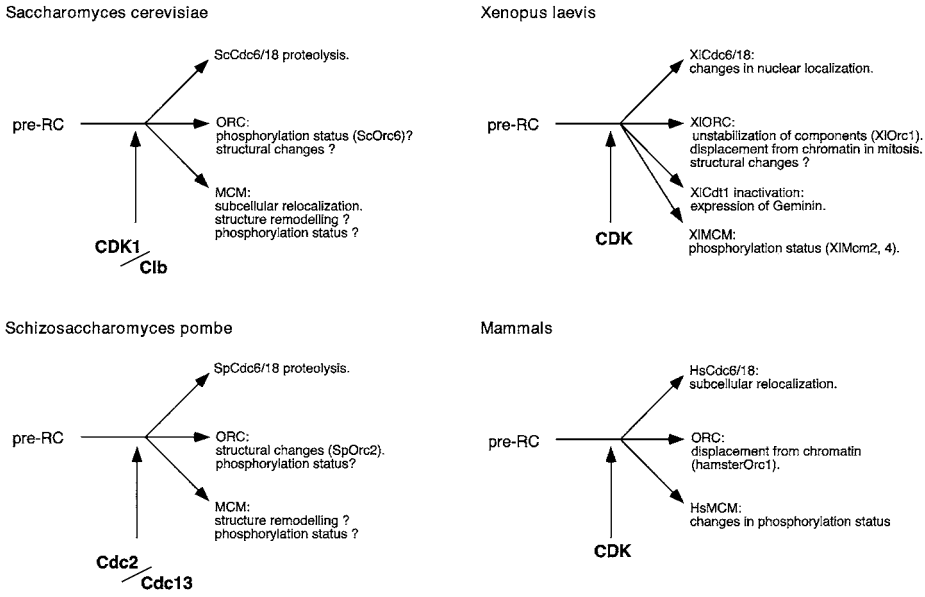


FIG. 14 Comparison of the CDK-mediated mechanisms controlling ploidy maintenance in budding yeast, fission yeast, *Xenopus,* and mammals.

absence of geminin is not sufficient to drive *Xenopus* cell extracts into an extra (and illegal) round of DNA replication (McGarry and Kirschner, 1998), indicating the existence of an overlapping control mechanism(s) in the maintenance of ploidy in frogs. Our current view of the ploidy maintenance mechanism in yeast and metazoans is summarized in Fig. 14.

VI. Concluding Remarks

Recent biochemical and genetic studies have provided a vast amount of information about how the initiation of DNA replication is organized in *S. cerevisiae*. Many, if not all, of the components, proteins, and DNA sequences involved in the initiation events in budding yeast have been identified: *cis*-acting elements at chromatin (ARSs), *trans*-acting DNA-interacting proteins known as initiators (ORC subunits, Cdc6, the Mcm2–7 family, Mcm10, and Cdc45), and regulators (S-CDK and DDK protein kinase complexes). Precise knowledge of the components required for replication initiation is allowing us to begin to understand step-by-step how cells hierarchically organize the different molecular events that lead to DNA synthesis from origins of replication. Important efforts have also been made toward the understanding of how this process is regulated in such a precise

way that eukaryotic cells timely replicate their DNA/genome only once during the cell division cycle. By identifying the targets of S-CDK and DDK kinases, we now have a full sketch of how this process might be regulated and, at the same time, how the machinery of the cell division cycle regulates the timing of initiation of DNA replication and elicits the control mechanism that ensure single rounds of DNA replication in each cell division. Nevertheless, many of the details are unknown. Also, the precise mechanism by which prereplicative complexes initiate the unwinding of the DNA at origins and the connection between initiation and the elongation machinery remain to be elucidated. Recent findings identified factors with a previously unknown role in the assembly of pre-RCs, such as Mcm10. Perhaps other factors remain to be identified. Furthermore, little is known about the (biochemical) role of each initiator in the assembly and disassembly of post- and pre-RC complexes. Several important questions remain to be answered, such as whether ORC ATPases activities play role in the conformational changes needed for the proper assembly of pre-RC complexes or in initiation events. Also, in terms of the proper timing of initiation, are these theoretical conformational changes somehow regulated by S-CDKs and DDK kinases? Since these kinases remain active from the G_1/S boundary to the end of mitosis, is the maintenance of these conformational changes important for preventing reassembly of pre-RCs throughout the cell cycle?

The current model of DNA replication initiation provides a comprehensive understanding of how unicellular eukaryotes avoid reassembly of pre-RC complexes at any given origin once DNA synthesis has been initiated. However, it fails to explain how rereplication is prevented from unfired origins of replication (those passively replicated). Although the inhibition mediated by Rad53 and Mec1 that prevents the firing of late origins when DNA replication is blocked suggests a mechanism for the prevention of initiation, little is known about pre-RCs disassembly at such origins or which are the substrates of these kinases among the initiators. Although it has been shown that the initiator protein Cdc6 has an ATPase domain required for its function, many biochemical details regarding the function of this enzymatic activity are far from complete. Recently, our group suggested that this initiator protein would act as a cyclin-dependent kinase inhibitor (CKI) cooperating with Sic1 and Hct1 in the inactivation of CDKs at the exit from mitosis. Since Cdc6 seems to be a CKI, might there be some relevant role for this activity in the assembly of prereplicative complexes? Might this Cdc6-mediated inhibition of CDK participate in the loading of Cdc6 onto ARSs–ORC complexes (post-RCs)? Several efforts are being directed toward *in vitro* analysis of the molecular events involved in the initiation of DNA replication, from the formation of an active pre-RC complex to the unwinding of the DNA. Such *in vitro* assays would help to explain the precise and hierarchical order and activities of each of the components involved—ARSs, ORC, Cdc6, MCMs, Cdc45, Cdt1, Mcm10, S-CDK, DDK, and perhaps Rad53 and Mec1 are the molecules already known to be initiators. Although indirect analyses clearly suggest that some of the

initiator proteins may be phosphorylated by kinase activators, direct evidence is needed. Thus, the question of what S-CDK and DDK substrates are involved in the initiation events and in the prevention of rereplication remains open. Similarly, the substrates of Rad53 and Mec1 function required for preventing the activation of late origins, although Orc2 may be one of them (as genetics suggests), remain obscure. As the molecular details of many elements participating in DNA replication initiation are revealed, new questions arise. Some of the future perspectives in research into DNA replication initiation have been addressed here, but certainly not all the pertinent questions have been raised.

Acknowledgments

We thank María Sacristán, Mar Sánchez, and members of the Sergio Moreno laboratory for helpful discussions and for critically reading the manuscript. We are grateful to Miguel Blanco for kindly providing the *S. pombe* photograph on Fig. 2. We also thank N. Skinner for correcting the English text.

References

Abdurashidova, G., Riva, S., Biamonti, G., Giacca, M., and Falaschi, A. (1998). Cell cycle modulation of protein–DNA interactions at a human replication origin. *EMBO J.* **17,** 2961–2969.

Aerne, B. L., Johnson, A. L., Toyn, J. H., and Johnston, L. H. (1998). Swi5 controls a novel wave of cyclin synthesis in late mitosis. *Mol. Biol. Cell* **9,** 945–956.

Alexandru, G., Zachariae, W., Schleiffer, A., and Nasmyth, K. (1999). Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *EMBO J.* **18,** 2707–2721.

Amon, A., Surana, U., Muroff, I., and Nasmyth, K. (1992). Regulation of p34*CDC28* tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature* **355,** 368–371.

Amon, A., Tyers, M., Futcher, B., and Nasmyth, K. (1993). Mechanisms that help the yeast cell cycle clock tick: G_2 cyclins transcriptionally activate G_2 cyclins and repress G_1 cyclins. *Cell* **74,** 993–1007.

Amon, A., Irniger, S., and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G_2 cyclin proteolysis initiated at mitosis persists until the activation of G_1 cyclins in the next cycle. *Cell* **77,** 1037–1050.

Andrews, B., and Measday, V. (1998). The cyclin family of budding yeast: Abundant use of a good idea. *Trends Genet.* **14,** 66–72.

Andrews, B. J., and Moore, L. A. (1992). Interaction of the yeast Swi4 and Swi6 cell cycle regulatory proteins *in vitro*. *Proc. Natl. Acad. Sci. USA* **89,** 11852–11856.

Antequera, F., and Bird, A. (1993). Number of CpG islands and genes in human and mouse. *Proc. Natl. Acad. Sci. USA* **90,** 11995–11999.

Antequera, F., and Bird, A. (1999). CpG islands as genomic footprints of promoters that are associated with replication origins. *Curr. Biol.* **9,** R661–R667.

Aparicio, O. M., Weinstein, D. M., and Bell, S. P. (1997). Components and dynamics of DNA replication complexes in S. *cerevisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91,** 59–69.

Aparicio, O. M., Stout, A. M., and Bell, S. P. (1999). Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proc. Natl. Acad. Sci. USA* **96,** 9130–9135.

Aravind, L., and Landsman, D. (1998). AT-hook motifs identified in a wide variety of DNA-binding proteins. *Nucleic Acids Res.* **26,** 4413–4421.

Arellano, M., and Moreno, S. (1997). Regulation of CDK/cyclin complexes during the cell cycle. *Int. J. Biochem. Cell. Biol.* **29,** 559–573.

Asano, M., and Wharton, R. P. (1999). E2F mediates developmental and cell cycle regulation of *ORC1* in *Drosophila. EMBO J.* **18,** 2435–2448.

Aves, S. J., Tongue, N., Foster, A. J., and Hart, E. A. (1998). The essential *Schizosaccharomyces pombe cdc23* DNA replication gene shares structural and functional homology with the *Saccharomyces cerevisiae DNA43* (*MCM10*) gene. *Curr. Genet.* **34,** 164–171.

Baker, T. A., and Bell, S. P. (1998). Polymerases and the replisome: Machines within machines. *Cell* **92,** 295–305.

Basco, R. D., Segal, M. D., and Reed, S. I. (1995). Negative regulation of G_1 and G_2 by S-phase cyclins of *Saccharomyces cerevisiae. Mol. Cell. Biol.* **15,** 5030–5042.

Baumer, M., Braus, G. H., and Irniger, S. (2000). Two different modes of cyclin clb2 proteolysis during mitosis in *Saccharomyces cerevisiae. FEBS Lett.* **468,** 142–148.

Beach, D., Piper, M., and Shall, S. (1980). Isolation of chromosomal origins of replication in yeast. *Nature* **284,** 185–187.

Bell, S. P., and Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357,** 128–134.

Benton, B. K., Tinkelenberg, A. H., Jean, D., Plump, S. D., and Cross, F. R. (1993). Genetic analysis of Cln/Cdc28 regulation of cell morphogenesis in budding yeast. *EMBO J.* **12,** 5267–5275.

Bielinsky, A. K., and Gerbi, S. A. (1999). Chromosomal *ARS1* has a single leading strand start site. *Mol. Cell* **3,** 477–486.

Blow, J. J., and Laskey, R. A. (1988). A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* **332,** 546–548.

Booher, R. N., Deshaies, R. J., and Kirschner, M. W. (1993). Properties of *Saccharomyces cerevisiae weel* and its differential regulation of p34*CDC28* in response to G_1 and G_2 cyclins. *EMBO J.* **12,** 3417–3426.

Bousset, K., and Diffley, J. F. (1998). The Cdc7 protein kinase is required for origin firing during S phase. *Genes Dev.* **12,** 480–490.

Boynton, S., and Tully, T. (1992). *latheo,* a new gene involved in associative learning and memory in *Drosophila melanogaster,* identified from P element mutagenesis. *Genetics* **131,** 655–672.

Brewer, B. J., and Fangman, W. L. (1987). The localization of replication origins on *ARS* plasmids in *S. cerevisiae. Cell* **51,** 463–471.

Brizuela, L., Draetta, G., and Beach, D. (1987). p13*suc1* acts in the fission yeast cell division cycle as a component of the p34*cdc2* protein kinase. *EMBO J.* **6,** 3507–3514.

Broek, D., Bartlett, R., Crawford, K., and Nurse, P. (1991). Involvement of p34*cdc2* in establishing the dependency of S phase on mitosis. *Nature* **349,** 388–393.

Brown, G. W., and Kelly, T. J. (1998). Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. *J. Biol. Chem.* **273,** 22083–22090.

Brown, J. A., Holmes, S. G., and Smith, M. M. (1991). The chromatin structure of *Saccharomyces cerevisiae* autonomously replicating sequences changes during the cell division cycle. *Mol. Cell. Biol.* **11,** 5301–5311.

Bueno, A., and Russell, P. (1992). Dual functions of *CDC6:* A yeast protein required for DNA replication also inhibits nuclear division. *EMBO J.* **11,** 2167–2176.

Caddle, M. S., and Calos, M. P. (1994). Specific initiation at an origin of replication from *Schizosaccharomyces pombe. Mol. Cell. Biol.* **14,** 1796–1805.

Calzada, A., Sacribtain, M., Sanchez, E., and Bueno, A. (2001). Cdc6 cooperates with Fic1 and Hct1 to inactivate mitotic cyclin-dependent kinases. *Nature* **412,** 355–358.

Calzada, A., Sanchez, M., Sanchez, E., and Bueno, A. (2000). The stability of the Cdc6 protein is regulated by cyclin-dependent kinase/cyclin B complexes in *Saccharomyces cerevisiae. J. Biol. Chem.* **275,** 9734–9741.

Carpenter, P. B., Mueller, P. R., and Dunphy, W. G. (1996). Role for a *Xenopus* Orc2-related protein in controlling DNA replication. *Nature* **379,** 357–360.

The *C. elegans* sequencing Consortium (1998). Genome sequence of the nematode *C. elegans*: A platform for investigating biology. *Science* **282,** 2012–2018.

Celniker, S. E., Sweder, K., Srienc, F., Bailey, J. E., and Campbell, J. L. (1984). Deletion mutations affecting autonomously replicating sequence *ARS1* of *Saccharomyces cerevisiae. Mol. Cell. Biol.* **4,** 2455–2466.

Chan, C. S., and Tye, B. K. (1980). Autonomously replicating sequences in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **77,** 6329–6333.

Chapman, J. W., and Johnston, L. H. (1989). The yeast gene, *DBF4,* essential for entry into S phase is cell cycle regulated. *Exp. Cell. Res.* **180,** 419–428.

Cheng, L., Collyer, T., and Hardy, C. F. (1999). Cell cycle regulation of DNA replication initiator factor Dbf4p. *Mol. Cell. Biol.* **19,** 4270–4278.

Chesnokov, I., Gossen, M., Remus, D., and Botchan, M. (1999). Assembly of functionally active *Drosophila* origin recognition complex from recombinant proteins. *Genes Dev.* **13,** 1289–1296.

Chong, J. P., Thommes, P., and Blow, J. J. (1996). The role of MCM/P1 proteins in the licensing of DNA replication. *Trends Biochem. Sci.* **21,** 102–106.

Chuang, R. Y., and Kelly, T. J. (1999). The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks. *Proc. Natl. Acad. Sci. USA* **96,** 2656–2661.

Cismowski, M. J., Laff, G. M., Solomon, M. J., and Reed, S. I. (1995). *KIN28* encodes a C-terminal domain kinase that controls mRNA transcription in *Saccharomyces cerevisiae* but lacks cyclin-dependent kinase-activating kinase (CAK) activity. *Mol. Cell. Biol.* **15,** 2983–2992.

Clyne, R. K., and Kelly, T. J. (1995). Genetic analysis of an *ARS* element from the fission yeast *Schizosaccharomyces pombe. EMBO J.* **14,** 6348–6357.

Cocker, J. H., Piatti, S., Santocanale, C., Nasmyth, K., and Diffley, J. F. (1996). An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* **379,** 180–182.

Coleman, T. R., Carpenter, P. B., and Dunphy, W. G. (1996). The *Xenopus* Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87,** 53–63.

Cooper, K. F., Mallory, M. J., Egeland, D. B., Jarnik, M., and Strich, R. (2000). Ama1p is a meiosis-specific regulator of the anaphase promoting complex/cyclosome in yeast. *Proc. Natl. Acad. Sci. USA* **97,** 14548–14553.

Correa-Bordes, J., and Nurse, P. (1995). p25*rum1* orders S phase and mitosis by acting as an inhibitor of the p34*cdc2* mitotic kinase. *Cell* **83,** 1001–1009.

Costanzo, M. C., Hogan, J. D., Cusick, M. E., Davis, B. P., Fancher, A. M., Hodges, P. E., Kondu, P., Lengieza, C., Lew-Smith, J. E., Lingner, C., Roberg-Perez, K. J., Tillberg, M. Brooks, J. E., and Garrels, J. I. (2000). The yeast proteome database (YPD) and *Caenorhabditis elegans* proteome database (WormPD): Comprehensive resources for the organization and comparison of model organism protein information. *Nucleic Acids Res.* **28,** 73–76.

Cross, F. R. (1988). *DAF1,* a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae. Mol. Cell. Biol.* **8,** 4675–4684.

Cross, F. R. (1990). Cell cycle arrest caused by *CLN* gene deficiency in *Saccharomyces cerevisiae* resembles START-I arrest and is independent of the mating–pheromone signalling pathway. *Mol. Cell. Biol.* **10,** 6482–6490.

Cross, F. R., and Blake, C. M. (1993). The yeast Cln3 protein is an unstable activator of Cdc28. *Mol. Cell. Biol.* **13,** 3266–3271.

Cross, F. R., and Levine, K. (1998). Molecular evolution allows bypass of the requirement for activation loop phosphorylation of the Cdc28 cyclin-dependent kinase. *Mol. Cell. Biol.* **18,** 2923–2931.

Cross, F. R., Hoek, M., McKinney, J. D., and Tinkelenberg, A. H. (1994). Role of Swi4 in cell cycle regulation of *CLN2* expression. *Mol. Cell. Biol.* **14,** 4779–4787.

Cvrckova, F., and Nasmyth, K. (1993). Yeast G1 cyclins *CLN1* and *CLN2* and a GAP-like protein have a role in bud formation. *EMBO J.* **12,** 5277–5286.

Dahmann, C., and Futcher, B. (1995). Specialization of B-type cyclins for mitosis or meiosis in *S. cerevisiae. Genetics* **140,** 957–963.

Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995). S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr. Biol.* **5,** 1257–1269.

Dalton, S., and Hopwood, B. (1997). Characterization of Cdc47p–minichromosome maintenance complexes in *Saccharomyces cerevisiae:* Identification of Cdc45p as a subunit. *Mol. Cell. Biol.* **17,** 5867–5875.

Dalton, S., and Whitbread, L. (1995). Cell cycle-regulated nuclear import and export of Cdc47, a protein essential for initiation of DNA replication in budding yeast. *Proc. Natl. Acad. Sci. USA* **92,** 2514–2518.

Delgado, S., Gomez, M., Bird, A., and Antequera, F. (1998). Initiation of DNA replication at CpG islands in mammalian chromosomes. *EMBO J.* **17,** 2426–2435.

DePamphilis, M. L. (1999). Replication origins in metazoan chromosomes: Fact or fiction? *Bioessays* **21,** 5–16.

Deshaies, R. J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell. Dev. Biol.* **15,** 435–467.

Deshaies, R. J., and Kirschner, M. (1995). G_1 cyclin-dependent activation of p34*CDC28* (Cdc28p) *in vitro. Proc. Natl. Acad. Sci. USA* **92,** 1182–1186.

Deshpande, A. M., and Newlon, C. S. (1992). The *ARS* consensus sequence is required for chromosomal origin function in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **12,** 4305–4313.

Detweiler, C. S., and Li, J. J. (1997). Cdc6p establishes and maintains a state of replication competence during G_1 phase. *J. Cell. Sci.* **110,** 753–763.

Detweiler, C. S., and Li, J. J. (1998). Ectopic induction of Clb2 in early G_1 phase is sufficient to block prereplicative complex formation in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **95,** 2384–2389.

Devin, A. B., Prosvirova, T., Peshekhonov, V. T., Chepurnaya, O. V., Smirnova, M. E., Koltovaya, N. A., Troitskaya, E. N., and Arman, I. P. (1990). The start gene *CDC28* and the genetic stability of yeast. *Yeast* **6,** 231–243.

Dhar, S. K., and Dutta, A. (2000). Identification and characterization of the human *ORC6* homolog. *J. Biol. Chem.* **275,** 34983–34988.

Diffley, J. F. (1998). Replication control: Choreographing replication origins. *Curr. Biol.* **8,** R771–R773.

Diffley, J. F., and Cocker, J. H. (1992). Protein–DNA interactions at a yeast replication origin. *Nature* **357,** 169–172.

Diffley, J. F., Cocker, J. H., Dowell, S. J., and Rowley, A. (1994). Two steps in the assembly of complexes at yeast replication origins *in vivo. Cell* **78,** 303–316.

Dirick, L., Bohm, T., and Nasmyth, K. (1995). Roles and regulation of Cln–Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae. EMBO J.* **14,** 4803–4813.

Dixon, W. J., and Campbell, J. L. (1997). Preparation of active Cdc7/Dbf4 kinase from yeast cells. *Methods Enzymol.* **283,** 390–397.

Donaldson, A. D., Fangman, W. L., and Brewer, B. J. (1998). Cdc7 is required throughout the yeast S phase to activate replication origins. *Genes Dev.* **12,** 491–501.

Donovan, J. D., Toyn, J. H., Johnson, A. L., and Johnston, L. H. (1994). P40*SDB25,* a putative CDK inhibitor, has a role in the M/G_1 transition in *Saccharomyces cerevisiae. Genes Dev.* **8,** 1640–1653.

Donovan, S., Harwood, J., Drury, L. S., and Diffley, J. F. (1997). Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl. Acad. Sci. USA* **94,** 5611–5616.

Dowell, S. J., Romanowski, P., and Diffley, J. F. (1994). Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins *in vivo. Science* **265,** 1243–1246.

Drury, L. S., Perkins, G., and Diffley, J. F. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J.* **16,** 5966–5976.

Drury, L. S., Perkins, G., and Diffley, J. F. (2000). The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr. Biol.* **10,** 231–240.

Dubey, D. D., Zhu, J., Carlson, D. L., Sharma, K., and Huberman, J. A. (1994). Three *ARS* elements contribute to the *ura4* replication origin region in the fission yeast, *Schizosaccharomyces pombe. EMBO J.* **13,** 3638–3647.

Dubey, D. D., Kim, S. M., Todorov, I. T., and Huberman, J. A. (1996). Large, complex modular structure of a fission yeast DNA replication origin. *Curr. Biol.* **6,** 467–473.

Ducommun, B., Brambilla, P., Felix, M. A., Franza, B. R., Karsenti, E., and Draetta, G. (1991). cdc2 phosphorylation is required for its interaction with cyclin. *EMBO J.* **10,** 3311–3319.

Dunphy, W. G., Brizuela, L., Beach, D., and Newport, J. (1988). The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54,** 423–431.

D'Urso, G., Marraccino, R. L., Marshak, D. R., and Roberts, J. M. (1990). Cell cycle control of DNA replication by a homologue from human cells of the p34*cdc2* protein kinase. *Science* **250,** 786–791.

Dutta, A., and Bell, S. P. (1997). Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell. Dev. Biol.* **13,** 293–332.

Dutta, A., and Stillman, B. (1992). cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. *EMBO J.* **11,** 2189–2199.

Dynlacht, B. D. (1997). Regulation of transcription by proteins that control the cell cycle. *Nature* **389,** 149–152.

Eki, T., Okumura, K., Amin, A., Ishiai, M., Abe, M., Nogami, M., Taguchi, H., Hurwitz, J., Murakami, Y., and Hanaoka, F. (1996). Mapping of the human homologue (*ORCIL*) of the yeast origin recognition complex subunit 1 gene to chromosome band 1p32. *Genomics* **36,** 559–561.

Elsasser, S., Lou, F., Wang, B., Campbell, J. L., and Jong, A. (1996). Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol. Biol. Cell* **7,** 1723–1735.

Elsasser, S., Chi, Y., Yang, P., and Campbell, J. L. (1999). Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis. *Mol. Biol. Cell* **10,** 3263–3277.

Epstein, C. B., and Cross, F. R. (1992). *CLB5:* A novel B cyclin from budding yeast with a role in S phase. *Genes Dev.* **6,** 1695–1706.

Espinoza, F. H., Farrell, A., Erdjument-Bromage, H., Tempst, P., and Morgan, D. O. (1996). A cyclin-dependent kinase-activating kinase (*CAK*) in budding yeast unrelated to vertebrate *CAK. Science* **273,** 1714–1717.

Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33,** 389–396.

Feger, G., Vaessin, H., Su, T. T., Wolff, E., Jan, L. Y., and Jan, Y. N. (1995). dpa, a member of the MCM family, is required for mitotic DNA replication but not endoreplication in *Drosophila. EMBO J.* **14,** 5387–5398.

Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91,** 221–230.

Ferreira, M. F., Santocanale, C., Drury, L. S., and Diffley, J. F. (2000). Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. *Mol. Cell. Biol.* **20,** 242–248.

Fitch, I., Dahmann, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., Byers, B., and Futcher, B. (1992). Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae. Mol. Biol. Cell* **3,** 805–818.

Foiani, M., Lucchini, G., and Plevani, P. (1997). The DNA polymerase alpha-primase complex couples DNA replication, cell-cycle progression and DNA-damage response. *Trends Biochem. Sci.* **22,** 424–427.

Ford, C., and Chevalier, S. (1995). DNA replication. Almost licensed. *Curr. Biol.* **5,** 1009–1012.

Forsburg, S. L., and Nurse, P. (1994). The fission yeast *cdc19+* gene encodes a member of the MCM family of replication proteins. *J. Cell Sci.* **107,** 2779–2788.

Fox, C. A., Loo, S., Dillin, A., and Rine, J. (1995). The origin recognition complex has essential functions in transcriptional silencing and chromosomal replication. *Genes Dev.* **9,** 911–924.

Friedman, K. L., Brewer, B. J., and Fangman, W. L. (1997). Replication profile of *Saccharomyces cerevisiae* chromosome VI. *Genes Cells* **2,** 667–678.

Fujita, M. (1999). Cell cycle regulation of DNA replication initiation proteins in mammalian cells. *Front. Biosci.* **4,** D816–D823.

Fujita, M., Yamada, C., Tsurumi, T., Hanaoka, F., Matsuzawa, K., and Inagaki, M. (1998). Cell cycle- and chromatin binding state-dependent phosphorylation of human MCM heterohexameric complexes. A role for cdc2 kinase. *J. Biol. Chem.* **273,** 17095–17101.

Gavin, K. A., Hidaka, M., and Stillman, B. (1995). Conserved initiator proteins in eukaryotes. *Science* **270,** 1667–1671.

Ghiara, J. B., Richardson, H. E., Sugimoto, K., Henze, M., Lew, D. J., Wittenberg, C., and Reed, S. I. (1991). A cyclin B homolog in *S. cerevisiae:* Chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. *Cell* **65,** 163–174.

Giacca, M., Zentilin, L., Norio, P., Diviacco, S., Dimitrova, D., Contreas, G., Biamonti, G., Perini, G., Weighardt, F., Riva, S., *et al.* (1994). Fine mapping of a replication origin of human DNA. *Proc. Natl. Acad. Sci. USA* **91,** 7119–7123.

Gibson, S. I., Surosky, R. T., and Tye, B. K. (1990). The phenotype of the minichromosome maintenance mutant *mcm3* is characteristic of mutants defective in DNA replication. *Mol. Cell. Biol.* **10,** 5707–5720.

Gilbert, D. M. (1998). Replication origins in yeast versus metazoa: Separation of the haves and the have nots. *Curr. Opin. Genet. Dev.* **8,** 194–199.

Gómez, M., and Antequera, F. (1999). Organization of DNA replication origins in the fission yeast genome. *EMBO J.* **18,** 5683–5690.

Gossen, M., Pak, D. T., Hansen, S. K., Acharya, J. K., and Botchan, M. R. (1995). A *Drosophila* homolog of the yeast origin recognition complex. *Science* **270,** 1674–1677.

Gould, K. L., Moreno, S., Owen, D. J., Sazer, S., and Nurse, P. (1991). Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34*cdc2* function. *EMBO J.* **10,** 3297–3309.

Grallert, B., and Nurse, P. (1996). The *ORC1* homolog *orp1* in fission yeast plays a key role in regulating onset of S phase. *Genes Dev.* **10,** 2644–2654.

Grandin, N., and Reed, S. I. (1993). Differential function and expression of *Saccharomyces cerevisiae* B-type cyclins in mitosis and meiosis. *Mol. Cell. Biol.* **13,** 2113–2125.

Guttes, E., and Guttes, S. (1969). Replication of nucleolus-associated DNA during "G_2 phase" in *Physarum polycephalum. J. Cell. Biol.* **43,** 229–236.

Hadwiger, J. A., Wittenberg, C., Mendenhall, M. D., and Reed, S. I. (1989a). The *Saccharomyces cerevisiae CKS1* gene, a homolog of the *Schizosaccharomyces pombe suc1+* gene, encodes a subunit of the Cdc28 protein kinase complex. *Mol. Cell. Biol.* **9,** 2034–2041.

Hadwiger, J. A., Wittenberg, C., Richardson, H. E., de Barros Lopes, M., and Reed, S. I. (1989b). A family of cyclin homologs that control the G_1 phase in yeast. *Proc. Natl. Acad. Sci. USA* **86,** 6255–6259.

Hardy, C. F. (1997). Identification of Cdc45p, an essential factor required for DNA replication. *Gene* **187,** 239–246.

Hardy, C. F., Dryga, O., Seematter, S., Pahl, P. M., and Sclafani, R. A. (1997). *mcm5/cdc46-bob1* bypasses the requirement for the S phase activator Cdc7p. *Proc. Natl. Acad. Sci. USA* **94,** 3151–3155.

Hartwell, L. H. (1973). Three additional genes required for deoxyribonucleic acid synthesis in *Saccharomyces cerevisiae. J. Bacteriol.* **115,** 966–974.

Hartwell, L. H., Culotti, J., and Reid, B. (1970). Genetic control of the cell-division cycle in yeast. I. Detection of mutants. *Proc. Natl. Acad. Sci. USA* **66,** 352–359.

Hartwell, L. H. (1976). Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. *J. Mol. Biol.* **104,** 803–817.

Hartwell, L. H. (1978). Cell division from a genetic perspective. *J. Cell Biol.* **77,** 627–637.

Hartwell, L. H., Culotti, J., Pringle, J. R., and Reid, B. J. (1974). Genetic control of the cell division cycle in yeast. *Science* **183,** 46–51.

Harvey, C. B., Wang, Y., Darmoul, D., Phillips, A., Mantei, N., and Swallow, D. M. (1996). Characterisation of a human homologue of a yeast cell division cycle gene, *MCM6,* located adjacent to the 5′ end of the lactase gene on chromosome 2q21. *FEBS Lett.* **398,** 135–140.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian *CDC6* is dependent on *E2F. Mol. Cell. Biol.* **18,** 6679–6697.

Hayles, J., Fisher, D., Woollard, A., and Nurse, P. (1994). Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34*cdc2*–mitotic B cyclin complex. *Cell* **78,** 813–822.

Heintz, N. H., and Hamlin, J. L. (1982). An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. *Proc. Natl. Acad. Sci. USA* **79,** 4083–4087.

Heinzel, S. S., Krysan, P. J., Tran, C. T., and Calos, M. P. (1991). Autonomous DNA replication in human cells is affected by the size and the source of the DNA. *Mol. Cell. Biol.* **11,** 2263–2272.

Hendrickson, M., Madine, M., Dalton, S., and Gautier, J. (1996). Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. *Proc. Natl. Acad. Sci. USA* **93,** 12223–12228.

Hennessy, K. M., Clark, C. D., and Botstein, D. (1990). Subcellular localization of yeast *CDC46* varies with the cell cycle. *Genes Dev.* **4,** 2252–2263.

Hennessy, K. M., Lee, A., Chen, E., and Botstein, D. (1991). A group of interacting yeast DNA replication genes. *Genes Dev.* **5,** 958–969.

Herendeen, D. R., and Kelly, T. J. (1996). DNA polymerase III: Running rings around the fork. *Cell* **84,** 5–8.

Hindley, J., and Phear, G. A. (1984). Sequence of the cell division gene *CDC2* from *Schizosaccharomyces pombe;* Patterns of splicing and homology to protein kinases. *Gene* **31,** 129–134.

Hindley, J., Phear, G., Stein, M., and Beach, D. (1987). *Suc1+* encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe. Mol. Cell. Biol.* **7,** 504–511.

Hofmann, J. F., and Beach, D. (1994). *cdt1* is an essential target of the Cdc10/Sct1 transcription factor: Requirement for DNA replication and inhibition of mitosis. *EMBO J.* **13,** 425–434.

Hogan, E., and Koshland, D. (1992). Addition of extra origins of replication to a minichromosome suppresses its mitotic loss in *cdc6* and *cdc14* mutants of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **89,** 3098–3102.

Hollingsworth, R. E., and Sclafani, R. A. (1990). DNA metabolism gene *CDC7* from yeast encodes a serine (threonine) protein kinase. *Proc. Natl. Acad. Sci. USA* **87,** 6272–6276.

Homesley, L., Lei, M., Kawasaki, Y., Sawyer, S., Christensen, T., and Tye, B. K. (2000). Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. *Genes Dev.* **14,** 913–926.

Hopwood, B., and Dalton, S. (1996). Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. *Proc. Natl. Acad. Sci. USA* **93,** 12309–12314.

Hori, Y., Shirahige, K., Obuse, C., Tsurimoto, T., and Yoshikawa, H. (1996). Characterization of a novel *CDC* gene (*ORC1*) partly homologous to *CDC6* of *Saccharomyces cerevisiae. Mol. Biol. Cell* **7,** 409–418.

Hu, B., Burkhart, R., Schulte, D., Musahl, C., and Knippers, R. (1993). The P1 family: A new class of nuclear mammalian proteins related to the yeast Mcm replication proteins. *Nucleic Acids Rec.* **21,** 5289–5293.

Hua, X. H., Yan, H., and Newport, J. (1997). A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. *J. Cell Biol.* **137,** 183–192.

Huang, R. Y., and Kowalski, D. (1993). A DNA unwinding element and an *ARS* consensus comprise a replication origin within a yeast chromosome. *EMBO J.* **12,** 4521–4531.

Huberman, J. A., Spotila, L. D., Nawotka, K. A., el-Assouli, S. M., and Davis, L. R. (1987). The *in vivo* replication origin of the yeast 2 microns plasmid. *Cell* **51,** 473–481.

Ishiai, M., Dean, F. B., Okumura, K., Abe, M., Moon, K. Y., Amin, A. A., Kagotani, K., Taguchi, H., Murakami, Y., Hanaoka, F., O'Donnell, M., Hurwitz, J., and Eki, T. (1997). Isolation of human and fission yeast homologues of the budding yeast origin recognition complex subunit ORC5: Human homologue (*ORC5L*) maps to 7q22. *Genomics* **46,** 294–298.

Ishimi, Y. (1997). A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. *J. Biol. Chem.* **272,** 24508–24513.

Ishimi, Y., Komamura, Y., You, Z., and Kimura, H. (1998). Biochemical function of mouse minichromosome maintenance 2 protein. *J. Biol. Chem.* **273,** 8369–8375.

Itzhaki, J. E., Gilbert, C. S., and Porter, A. C. (1997). Construction by gene targeting in human cells of a "conditional' *CDC2* mutant that rereplicates its DNA. *Nat. Genet* **15,** 258–265.

Izumi, M., Yanagi, K., Mizuno, T., Yokoi, M., Kawasaki, Y., Moon, K. Y., Hurwitz, J., Yatagai, F., and Hanaoka, F. (2000). The human homolog of *Saccharomyces cerevisiae* Mcm10 interacts with replication factors and dissociates from nuclease-resistant nuclear structures in G(2) phase. *Nucleic Acids Res.* **28,** 4769–4777.

Jackson, A. L., Pahl, P. M., Harrison, K., Rosamond, J., and Sclafani, R. A. (1993). Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. *Mol. Cell. Biol.* **13,** 2899–2908.

Jallepalli, P. V., and Kelly, T. J. (1996). Rum1 and Cdc18 link inhibition of cyclin-dependent kinase to the initiation of DNA replication in *Schizosaccharomyces pombe. Genes Dev.* **10,** 541–552.

Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D., and Kelly, T. J. (1997). Regulation of the replication initiator protein p65*cdc18* by CDK phosphorylation. *Genes Dev.* **11,** 2767–2779.

Jallepalli, P. V., Tien, D., and Kelly, T. J. (1998). *sud1*(+) targets cyclin-dependent kinase-phosphorylated Cdc18 and Rum1 proteins for degradation and stops unwanted diploidization in fission yeast. *Proc. Natl. Acad. Sci. USA* **95,** 8159–8164.

Jaspersen, S. L., Charles, J. F., and Morgan, D. O. (1999). Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. *Curr. Biol.* **9,** 227–236.

Jiang, W., and Hunter, T. (1997). Identification and characterization of a human protein kinase related to budding yeast Cdc7p. *Proc. Natl. Acad. Sci. USA* **94,** 14320–14325.

Johnston, G. C. (1977). Cell size and budding during starvation of the yeast *Saccharomyces cerevisiae. J. Bacteriol.* **132,** 738–739.

Johnston, L. H., and Thomas, A. P. (1982). A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae. Mol. Gen. Genet.* **186,** 445–448.

Johnston, L. H., Masai, H., and Sugino, A. (1999). First the CDKs, now the DDKs. *Trends Cell. Biol.* **9,** 249–252.

Kaldis, P., Sutton, A., and Solomon, M. J. (1996). The Cdk-activating kinase (CAK) from budding yeast. *Cell* **86,** 553–564.

Kearsey, S. E., and Labib, K. (1998). MCM proteins: Evolution, properties, and role in DNA replication. *Biochim. Biophys. Acta* **1398,** 113–136.

Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1993). The fission yeast *cdc18*+ gene product couples S phase to START and mitosis. *Cell* **74,** 371–382.

Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kawasaki, Y., Johnston, L. H., and Sugino, A. (2000). Characterization of the yeast Cdc7p/Dbf4p complex purified from insect Cells. Its protein kinase activity is regulated by Rad53p. *J. Biol. Chem.* **275,** 35051–35062.

Kim, J. M., Sato, N., Yamada, M., Arai, K., and Masai, H. (1998). Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to *Saccharomyces cerevisiae CDC7* essential for G_1/S transition. Structure, chromosomal localization, and expression of mouse gene for *S. cerevisiae* Cdc7-related kinase. *J. Biol. Chem.* **273,** 23248–23257.

Kim, S. M., and Huberman, J. A. (1998). Multiple orientation-dependent, synergistically interacting, similar domains in the ribosomal DNA replication origin of the fission yeast, *Schizosaccharomyces pombe. Mol. Cell. Biol.* **18,** 7294–7303.

Kimura, H., Nozaki, N., and Sugimoto, K. (1994). DNA polymerase alpha associated protein P1, a murine homolog of yeast *MCM3,* changes its intranuclear distribution during the DNA synthetic period. *EMBO J.* **13,** 4311–4320.

Kimura, H., Takizawa, N., Nozaki, N., and Sugimoto, K. (1995). Molecular cloning of cDNA encoding mouse *Cdc21* and *CDC46* homologs and characterization of the products: Physical interaction between P1(MCM3) and CDC46 proteins. *Nucleic Acids Res.* **23,** 2097–2104.

Kimura, H., Ohtomo, T., Yamaguchi, M., Ishii, A., and Sugimoto, K. (1996). Mouse MCM proteins: Complex formation and transportation to the nucleus. *Genes Cells* **1,** 977–993.

King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. (1996). How proteolysis drives the cell cycle. *Science* **274,** 1652–1659.

Kingsman, A. J., Clarke, L., Mortimer, R. K., and Carbon, J. (1979). Replication in *Saccharomyces cerevisiae* of plasmid pBR313 carrying DNA from the yeast *trpl* region. *Gene* **7,** 141–152.

Kitada, K., Johnson, A. L., Johnston, L. H., and Sugino, A. (1993). A multicopy suppressor gene of the *Saccharomyces cerevisiae* G_1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5. Mol. Cell. Biol.* **13,** 4445–4457.

Kitsberg, D., Selig, S., Keshet, I., and Cedar, H. (1993). Replication structure of the human beta-globin gene domain. *Nature* **366,** 588–590.

Kiyono, T., Fujita, M., Hayashi, Y., and Ishibashi, M. (1996). Cloning of a cDNA encoding a human homologue of *CDC47,* a member of the MCM family. *Biochim. Biophys. Acta* **1307,** 31–34.

Klemm, R. D., Austin, R. J., and Bell, S. P. (1997). Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* **88,** 493–502.

Knapp, D., Bhoite, L., Stillman, D. J., and Nasmyth, K. (1996). The transcription factor Swi5 regulates expression of the cyclin kinase inhibitor p40*SIC1*. *Mol. Cell. Biol.* **16,** 5701–5707.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* **77,** 107–120.

Koch, C., Moll, T., Neuberg, M., Ahorn, H., and Nasmyth, K. (1993). A role for the transcription factors Mbp1 and Swi4 in progression from G_1 to S phase. *Science* **261,** 1551–1557.

Kominami, K., and Toda, T. (1997). Fission yeast WD-repeat protein pop1 regulates genome ploidy through ubiquitin-proteasome-mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. *Genes Dev.* **11,** 1548–1560.

Kominami, K., Ochotorena, I., and Toda, T. (1998). Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero- and homo- complexes together with cullin-1 in the fission yeast SCF (Skp1–Cullin–1-F-box) ubiquitin ligase. *Genes Cells* **3,** 721–735.

Koonin, E. V. (1993). A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. *Nucleic Acids Res.* **21,** 2541–2547.

Krude, T., Jackman, M., Pines, J., and Laskey, R. A. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* **88,** 109–119.

Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H., and Nojima, H. (1995). Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA replication licensing factor. *Cell* **81,** 601–609.

Kuhne, C., and Linder, P. (1993). A new pair of B-type cyclins from *Saccharomyces cerevisiae* that function early in the cell cycle. *EMBO J.* **12,** 3437–3447.

Kumagai, H., Sato, N., Yamada, M., Mahony, D., Seghezzi, W., Lees, E., Arai, K., and Masai, H. (1999). A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G_1/S transition in mammalian cells. *Mol. Cell. Biol.* **19,** 5083–5095.

Labib, K., Diffley, J. F., and Kearsey, S. E. (1999). G_1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat. Cell. Biol.* **1,** 415–422.

Labib, K., Tercero, J. A., and Diffley, J. F. (2000). Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science* **288,** 1643–1467.

Landis, G., and Tower, J. (1999). The *Drosophila chiffon* gene is required for chorion gene amplification, and is related to the yeast Dbf4 regulator of DNA replication and cell cycle. *Development* **126,** 4281–4293.

Lanker, S., Valdivieso, M. H., and Wittenberg, C. (1996). Rapid degradation of the G_1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science* **271,** 1597–1601.

Larsen, F., Gundersen, G., Lopez, R., and Prydz, H. (1992). CpG islands as gene markers in the human genome. *Genomics* **13,** 1095–1107.

Leatherwood, J. (1998). Emerging mechanisms of eukaryotic DNA replication initiation. *Curr. Opin. Cell Biol.* **10,** 742–748.

Leatherwood, J., Lopez-Girona, A., and Russell, P. (1996). Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. *Nature* **379,** 360–363.

Lee, D. G., and Bell, S. P. (1997). Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol. Cell. Biol.* **17,** 7159–7168.

Lee, D. G., and Bell, S. P. (2000). ATPase switches controlling DNA replication initiation. *Curr. Opin. Cell Biol.* **12,** 280–285.

Lee, J. K., and Hurwitz, J. (2000). Isolation and characterization of various complexes of the minichromosome maintenance proteins of *Schizosaccharomyces pombe. J. Biol. Chem.* **275,** 18871–18878.

Lehner, C. F., and O'Farrell, P. H. (1990). The roles of *Drosophila* cyclins A and B in mitotic control. *Cell* **61,** 535–547.

Lei, M., Kawasaki, Y., and Tye, B. K. (1996). Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **16,** 5081–5090.

Lei, M., Kawasaki, Y., Young, M. R., Kihara, M., Sugino, A., and Tye, B. K. (1997). Mcm2 is a target of regulation by Cdc7–Dbf4 during the initiation of DNA synthesis. *Genes Dev.* **11,** 3365–3374.

Lepke, M., Putter, V., Staib, C., Kneissl, M., Berger, C., Hoehn, K., Nanda, I., Schmid, M., and Grummt, F. (1999). Identification, characterization and chromosomal localization of the cognate human and murine *DBF4* genes. *Mol. Gen. Genet.* **262,** 220–229.

Levine, K., Huang, K., and Cross, F. R. (1996). *Saccharomyces cerevisiae* G_1 cyclins differ in their intrinsic functional specificities. *Mol. Cell. Biol.* **16,** 6794–6803.

Lew, D. J., and Kornbluth, S. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. *Curr. Opin. Cell Biol.* **8,** 795–804.

Lew, D. J., and Reed, S. I. (1992). A proliferation of cyclins. *Trends Cell Biol.* **2,** 77–81.

Lew, D. J., and Reed, S. I. (1993). Morphogenesis in the yeast cell cycle: Regulation by Cdc28 and cyclins. *J. Cell Biol.* **120,** 1305–1320.

Li, J. J., and Herskowitz, I. (1993). Isolation of *ORC6,* a component of the yeast origin recognition complex by a one-hybrid system. *Science* **262,** 1870–1874.

Liang, C., and Stillman, B. (1997). Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in *cdc6* mutants. *Genes Dev.* **11,** 3375–3386.

Liang, C., Weinreich, M., and Stillman, B. (1995). ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81,** 667–676.

Lim, H. H., Goh, P. Y., and Surana, U. (1996a). Spindle pole body separation in *Saccharomyces cerevisiae* requires dephosphorylation of the tyrosine 19 residue of Cdc28. *Mol. Cell. Biol.* **16,** 6385–6397.

Lim, H. H., Loy, C. J., Zaman, S., and Surana, U. (1996b). Dephosphorylation of threonine 169 of Cdc28 is not required for exit from mitosis but may be necessary for START in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **16,** 4573–4583.

Loebel, D., Huikeshoven, H., and Cotterill, S. (2000). Localisation of the DmCdc45 DNA replication factor in the mitotic cycle and during chorion gene amplification. *Nucleic Acids Res.* **28,** 3897–3903.

Lohka, M. J., Hayes, M. K., and Maller, J. L. (1988). Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. *Proc. Natl. Acad. Sci. USA* **85,** 3009–3013.

Loo, S., Fox, C. A., Rine, J., Kobayashi, R., Stillman, B., and Bell, S. (1995). The origin recognition complex in silencing, cell cycle progression, and DNA replication. *Mol. Biol. Cell* **6,** 741–756.

López-Girona, A., Mondesert, O., Leatherwood, J., and Russell, P. (1998). Negative regulation of Cdc18 DNA replication protein by Cdc2. *Mol. Biol. Cell* **9,** 63–73.

Lorincz, A. T., and Reed, S. I. (1984). Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. *Nature* **307,** 183–185.

Lygerou, Z., and Nurse, P. (1999). The fission yeast origin recognition complex is constitutively associated with chromatin and is differentially modified through the cell cycle. *J. Cell Sci.* **112,** 3703–3712.

MacNeill, S. A., and Nurse, P. (1997). Cell cycle control in fission yeast. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell Cycle and Cell Biology* (J. R. Pringle, J. R. Broach, and E. W. Jones, Eds.), pp. 697–763. CSHL, New York.

MacNeill, S. A., Creanor, J., and Nurse, P. (1991). Isolation, characterisation and molecular cloning of new mutant alleles of the fission yeast p34*cdc2*+ protein kinase gene: Identification of temperature-sensitive G_2 arresting alleles. *Mol. Gen. Genet.* **229,** 109–118.

Mahbubani, H. M., Chong, J. P., Chevalier, S., Thommes, P., and Blow, J. J. (1997). Cell cycle regulation of the replication licensing system: Involvement of a Cdk-dependent inhibitor. *J. Cell Biol.* **136,** 125–135.

Maine, G. T., Sinha, P., and Tye, B. K. (1984). Mutants of *S. cerevisiae* defective in the maintenance of minichromosomes. *Genetics* **106,** 365–385.

Maiorano, D., Van Assendelft, G. B., and Kearsey, S. E. (1996). Fission yeast *cdc21,* a member of the MCM protein family, is required for onset of S phase and is located in the nucleus throughout the cell cycle. *EMBO J.* **15,** 861–872.

Maiorano, D., Moreau, J., and Mechali, M. (2000). *XCDT1* is required for the assembly of prereplicative complexes in *Xenopus laevis. Nature* **404,** 622–625.

Marahrens, Y., and Stillman, B. (1992). A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* **255,** 817–823.

Marahrens, Y., and Stillman, B. (1994). Replicator dominance in a eukaryotic chromosome. *EMBO J.* **13,** 3395–3400.

Masai, H., Miyake, T., and Arai, K. (1995). *hsk1*+, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae CDC7,* is required for chromosomal replication. *EMBO J.* **14,** 3094–3104.

Maundrell, K., Hutchison, A., and Shall, S. (1988). Sequence analysis of *ARS* elements in fission yeast. *EMBO J.* **7,** 2203–2209.

McGarry, T. J., and Kirschner, M. W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93,** 1043–1053.

McInerny, C. J., Partridge, J. F., Mikesell, G. E., Creemer, D. P., and Breeden, L. L. (1997). A novel Mcm1-dependent element in the *SWI4, CLN3, CDC6,* and *CDC47* promoters activates M/G_1-specific transcription. *Genes Dev.* **11,** 1277–1288.

McKinney, J. D., Chang, F., Heintz, N., and Cross, F. R. (1993). Negative regulation of *FAR1* at the START of the yeast cell cycle. *Genes Dev.* **7,** 833–843.

Mendenhall, M. D. (1993). An inhibitor of p34*CDC28* protein kinase activity from *Saccharomyces cerevisiae. Science* **259,** 216–219.

Mendenhall, M. D., and Hodge, A. E. (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev.* **62,** 1191–1243.

Merchant, A. M., Kawasaki, Y., Chen, Y., Lei, M., and Tye, B. K. (1997). A lesion in the DNA replication initiation factor Mcm 10 induces pausing of elongation forks through chromosomal replication origins in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **17,** 3261–3271.

Micklem, G., Rowley, A., Harwood, J., Nasmyth, K., and Diffley, J. F. (1993). Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. *Nature* **366,** 87–89.

Mimura, S., and Takisawa, H. (1998). *Xenopus* Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk. *EMBO J.* **17,** 5699–5707.

Miyake, S., and Yamashita, S. (1998). Identification of *sna41* gene, which is the suppressor of *nda4* mutation and is involved in DNA replication in *Schizosaccharomyces pombe. Genes Cells* **3,** 157–166.

Miyake, S., Okishio, N., Samejima, I., Hiraoka, Y., Toda, T., Saitoh, I., and Yanagida, M. (1993). Fission yeast genes *nda1*+ and *nda4*+, mutations of which lead to S-phase block, chromatin alteration and Ca^{2+} suppression, are members of the *CDC46/MCM2* family. *Mol. Biol. Cell* **4,** 1003–1015.

Miyake, S., Saito, I., Kobayashi, H., and Yamashita, S. (1996). Identification of two *Xenopus laevis* genes, *xMCM2* and *xCDC46,* with sequence homology to *MCM* genes involved in DNA replication. *Gene* **175,** 71–75.

Mizushima, T., Takahashi, N., and Stillman, B. (2000). Cdc6p modulates the structure and DNA binding activity of the origin recognition complex *in vitro. Genes Dev.* **14,** 1631–1641.

Moir, D., Stewart, S. E., Osmond, B. C., and Botstein, D. (1982). Cold-sensitive cell-division-cycle mutants of yeast: Isolation, properties, and pseudoreversion studies. *Genetics* **100,** 547–563.

Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor *SW15. Cell* **66,** 743–758.

Mondesert, O., McGowan, C. H., and Russell, P. (1996). Cig2, a B-type cyclin, promotes the onset of S in *Schizosaccharomyces pombe. Mol. Cell. Biol.* **16,** 1527–1533.

Moon, K. Y., Kong, D., Lee, J. K., Raychaudhuri, S., and Hurwitz, J. (1999). Identification and reconstitution of the origin recognition complex from *Schizosaccharomyces pombe. Proc. Natl. Acad. Sci. USA* **96,** 12367–12372.

Moreno, S., and Nurse, P. (1994). Regulation of progression through the G_1 phase of the cell cycle by the *rum1*+ gene. *Nature* **367,** 236–242.

Morgan, D. O. (1995). Principles of CDK regulation. *Nature* **374,** 131–134.

Musahl, C., Schulte, D., Burkhart, R., and Knippers, R. (1995). A human homologue of the yeast replication protein Cdc21. Interactions with other Mcm proteins. *Eur. J. Biochem.* **230,** 1096–1101.

Muzi-Falconi, M., and Kelly, T. J. (1995). Orp1, a member of the Cdc18/Cdc6 family of S-phase regulators, is homologous to a component of the origin recognition complex. *Proc. Natl. Acad. Sci. USA* **92,** 12475–12479.

Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, A. B. (1988). The *WHI1*+ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.* **7,** 4335–4346.

Nasheuer, H. P., Moore, A., Wahl, A. F., and Wang, T. S. (1991). Cell cycle-dependent phosphorylation of human DNA polymerase alpha. *J. Biol. Chem.* **266,** 7893–7903.

Nasmyth, K. (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.* **5,** 166–179.

Nasmyth, K. (1996). Viewpoint: Putting the cell cycle in order. *Science* **274,** 1643–1645.

Nasmyth, K., and Dirick, L. (1991). The role of *SWI4* and *SWI6* in the activity of G_1 cyclins in yeast. *Cell* **66,** 995–1013.

Nasmyth, K. A., and Reed, S. I. (1980). Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci. USA* **77,** 2119–2123.

Nasmyth, K., Adolf, G., Lydall, D., and Seddon, A. (1990). The identification of a second cell cycle control on the *HO* promoter in yeast: Cell cycle regulation of *SWI5* nuclear entry. *Cell* **62,** 631–647.

Natale, D. A., Li, C. J., Sun, W. H., and DePamphilis, M. L. (2000). Selective instability of Orc1 protein accounts for the absence of functional origin recognition complexes during the M-G(1) transition in mammals. *EMBO J.* **19,** 2728–2738.

Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9,** 27–43.

Newlon, C. S., and Theis, J. F. (1993). The structure and function of yeast *ARS* elements. *Curr. Opin. Genet. Dev.* **3,** 752–758.

Nguyen, V. Q., Co, C., Irie, K., and Li, J. J. (2000). Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7. *Curr. Biol.* **10,** 195–205.

Nguyen, V. Q., Co, C., and Li, J. J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature* **411,** 1068–1073.

Nishitani, H., and Nurse, P. (1995). p65*cdc18* plays a major role controlling the initiation of DNA replication is fission yeast. *Cell* **83,** 397–405.

Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* **404,** 625–628.

Noton, E., and Diffley, J. F. (2000). CDK inactivation is the only essential function of the APC/C and the mitotic exit network proteins for origin resetting during mitosis. *Mol. Cell* **5,** 85–95.

Nougarede, R., Della Seta, F., Zarzov, P., and Schwob, E. (2000). Hierarchy of S-phase-promoting factors: Yeast Dbf4–Cdc7 kinase requires prior S-phase cyclin-dependent kinase activation. *Mol. Cell. Biol.* **20,** 3795–3806.

Nurse, P. (1975). Genetic control of cell size at cell division in yeast. *Nature* **256,** 547–551.

Nurse, P., and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe. Genetics* **96,** 627–637.

Oehlen, L. J., and Cross, F. R. (1994). G_1 cyclins *CLN1* and *CLN2* repress the mating factor response pathway at START in the yeast cell cycle. *Genes Dev.* **8,** 1058–1070.

Oehlen, L. J., Jeoung, D. I., and Cross, F. R. (1998). Cyclin-specific START events and the G_1-phase specificity of arrest by mating factor in budding yeast. *Mol. Gen. Genet.* **258,** 183–198.

Ogas, J., Andrews, B. J., and Herskowitz, I. (1991). Transcriptional activation of *CLN1, CLN2,* and a putative new G_1 cyclin (*HCS26*) by *SWI4,* a positive regulator of G_1-specific transcription. *Cell* **66,** 1015–1026.

Ohno, K., Hirose, F., Inoue, Y. H., Takisawa, H., Mimura, S., Hashimoto, Y., Kiyono, T., Nishida, Y., and Matsukage, A. (1998). cDNA cloning and expression during development of *Drosophila melanogaster MCM3, MCM6* and *MCM7. Gene* **217,** 177–185.

Okishio, N., Adachi, Y., and Yanagida, M. (1996). Fission yeast Nda1 and Nda4, MCM homologs required for DNA replication, are constitutive nuclear proteins. *J. Cell Sci.* **109,** 319–326.

Okuno, Y., Okazaki, T., and Masukata, H. (1997). Identification of a predominant replication origin in fission yeast. *Nucleic Acids Res.* **25,** 530–537.

Pak, D. T., Pflumm, M., Chesnokov, I., Huang, D. W., Kellum, R., Marr, J., Romanowski, P., and Botchan, M. R. (1997). Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91,** 311–323.

Pasion, S. G., and Forsburg, S. L. (1999). Nuclear localization of *Schizosaccharomyces pombe* Mcm2/Cdc19p requires MCM complex assembly. *Mol. Biol. Cell* **10,** 4043–4057.

Patel, S., and Latterich, M. (1998). The AAA team: Related ATPases with diverse functions. *Trends Cell Biol.* **8,** 65–71.

Patterson, M., Sclafani, R. A., Fangman, W. L., and Rosamond, J. (1986). Molecular characterization of cell cycle gene *CDC7* from *Saccharomyces cerevisiae. Mol. Cell. Biol.* **6,** 1590–1598.

Paulovich, A. G., and Hartwell, L. H. (1995). A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell* **82,** 841–847.

Perkins, G., and Diffley, J. F. (1998). Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. *Mol. Cell* **2,** 23–32.

Peter, M., and Herskowitz, I. (1994a). Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265,** 1228–1231.

Peter, M., and Herskowitz, I. (1994b). Joining the complex: Cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell* **79,** 181–184.

Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *EMBO J.* **18,** 396–410.

Petersen, B. O., Wagener, C., Marinoni, F., Kramer, E. R., Melixetian, M., Denchi, E. L., Gieffers, C., Matteucci, C., Peters, J. M., and Helin, K. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. *Genes Dev.* **14,** 2330–2343.

Piatti, S., Lengauer, C., and Nasmyth, K. (1995). Cdc6 is an unstable protein whose *de novo* synthesis in G_1 is important for the onset of S phase and for preventing a "reductional" anaphase in the budding yeast *Saccharomyces cerevisiae. EMBO J.* **14,** 3788–3799.

Piatti, S., Bohm, T., Cocker, J. H., Diffley, J. F., and Nasmyth, K. (1996). Activation of S-phase-promoting CDKs in late G_1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. *Genes Dev.* **10,** 1516–1531.

Pierron, G., Pallotta, D., and Benard, M. (1999). The one-kilobase DNA fragment upstream of the *ardC* actin gene of *Physarum polycephalum* is both a replicator and a promoter. *Mol. Cell. Biol.* **19,** 3506–3514.

Piggott, J. R., Rai, R., and Carter, B. L. (1982). A bifunctional gene product involved in two phases of the yeast cell cycle. *Nature* **298,** 391–393.

Pinto, S., Quintana, D. G., Smith, P., Mihalek, R. M., Hou, Z. H., Boynton, S., Jones, C. J., Hendricks, M., Velinzon, K., Wohlschlegel, J. A., Austin, R. J., Lane, W. S., Tully, T., and Dutta, A. (1999). *latheo* encodes a subunit of the origin recognition complex and disrupts neuronal proliferation and adult olfactory memory when mutant. *Neuron* **23,** 45–54.

Primig, M., Sockanathan, S., Auer, H., and Nasmyth, K. (1992). Anatomy of a transcription factor important for the start of the cell cycle in *Saccharomyces cerevisiae. Nature* **358,** 593–597.

Quintana, D. G., Hou, Z., Thome, K. C., Hendricks, M., Saha, P., and Dutta, A. (1997). Identification of *HsORC4,* a member of the human origin of replication recognition complex. *J. Biol. Chem.* **272,** 28247–28251.

Quintana, D. G., Thome, K. C., Hou, Z. H., Ligon, A. H., Morton, C. C., and Dutta, A. (1998). *ORC5L,* a new member of the human origin recognition complex, is deleted in uterine leiomyomas and malignant myeloid diseases. *J. Biol. Chem.* **273,** 27137–27145.

Rao, H., and Stillman, B. (1995). The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. *Proc. Natl. Acad. Sci. USA* **92,** 2224–2228.

Rao, H., Marahrens, Y., and Stillman, B. (1994). Functional conservation of multiple elements in yeast chromosomal replicators. *Mol. Cell. Biol.* **14,** 7643–7651.

Rao, P. N., and Johnson, R. T. (1970). Mammalian cell fusion: Studies on the regulation of DNA synthesis and mitosis. *Nature* **225,** 159–164.

Reed, S. I., and Wittenberg, C. (1990). Mitotic role for the Cdc28 protein kinase of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **87,** 5697–5701.

Reid, R. J., Fiorani, P., Sugawara, M., and Bjornsti, M. A. (1999). CDC45 and DPB11 are required for processive DNA replication and resistance to DNA topoisomerase I-mediated DNA damage. *Proc. Natl. Acad. Sci. USA* **96,** 11440–11445.

Richardson, H. E., Wittenberg, C., Cross, F., and Reed, S. I. (1989). An essential G_1 function for cyclin-like proteins in yeast. *Cell* **59,** 1127–1133.

Richardson, H., Lew, D. J., Henze, M., Sugimoto, K., and Reed, S. I. (1992). Cyclin-B homologs in *Saccharomyces cerevisiae* function in S phase and in G_2. *Genes Dev.* **6,** 2021–2034.

Roberts, J. M., and D'Urso, G. (1988). An origin unwinding activity regulates initiation of DNA replication during mammalian cell cycle. *Science* **241,** 1486–1489.

Romanowski, P., Madine, M. A., and Laskey, R. A. (1996a). XMCM7, a novel member of the *Xenopus* MCM family, interacts with XMCM3 and colocalizes with it throughout replication. *proc. Natl. Acad. Sci. USA* **93,** 10189–10194.

Romanowski, P., Madine, M. A., Rowles, A., Blow, J. J., and Laskey, R. A. (1996b). The *Xenopus* origin recognition complex is essential for DNA replication and MCM binding to chromatin. *Curr. Biol.* **6,** 1416–1425.

Rowles, A., Chong, J. P., Brown, L., Howell, M., Evan, G. I., and Blow, J. J. (1996). Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell* **87,** 287–296.

Rowley, A., Dowell, S. J., and Diffley, J. F. (1994). Recent developments in the initiation of chromosomal DNA replication: A complex picture emerges. *Biochim. Biophys. Acta* **1217,** 239–256.

Rowley, A., Cocker, J. H., Harwood, J., and Diffley, J. F. (1995). Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. *EMBO J.* **14,** 2631–2641.

Russell, P., Moreno, S., and Reed, S. I. (1989). Conservation of mitotic controls in fission and budding yeasts. *Cell* **57,** 295–303.

Saha, P., Chen, J., Thome, K. C., Lawlis, S. J., Hou, Z. H., Hendricks, M., Parvin, J. D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol. Cell. Biol.* **18,** 2758–2767.

Sánchez, M., Calzada, A., and Bueno, A. (1999). The Cdc6 protein is ubiquitinated *in vivo* for proteolysis in *Saccharomyces cerevisiae. J. Biol. Chem.* **274,** 9092–9097.

Sánchez-Díaz, A., González, I., Arellano, M., and Moreno, S. (1998). The Cdk inhibitors p*25rum1* and p40*SIC1* are functional homologues that play similar roles in the regulation of the cell cycle in fission and budding yeast. *J. Cell Sci.* **111,** 843–851.

Santocanale, C., and Diffley, J. F. (1996). ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in *Saccharomyces cerevisiae. EMBO J.* **15,** 6671–6679.

Santocanale, C., and Diffley, J. F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* **395,** 615–618.

Sasaki, T., Sawado, T., Yamaguchi, M., and Shinomiya, T. (1999). Specification of regions of DNA replication initiation during embryogenesis in the 65-kilobase DNApolalpha-dE2F locus of *Drosophila melanogaster. Mol. Cell. Biol.* **19,** 547–555.

Sato, N., Arai, K., and Masai, H. (1997). Human and *Xenopus* cDNAs encoding budding yeast Cdc7-related kinases: *In vitro* phosphorylation of MCM subunits by a putative human homologue of Cdc7. *EMBO J.* **16,** 4340–4351.

Sauer, K., Knoblich, J. A., Richardson, H., and Lehner, C. F. (1995). Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of *Drosophila* embryogenesis. *Genes Dev.* **9,** 1327–1339.

Schulte, D., Richter, A., Burkhart, R., Musahl, C., and Knippers, R. (1996). Properties of the human nuclear protein p85Mcm. Expression, nuclear localization and interaction with other Mcm proteins. *Eur. J. Biochem.* **235,** 144–151.

Schwab, M., Lutum, A. S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell* **90,** 683–693.

Schwob, E., and Nasmyth, K. (1993). *CLB5* and *CLB6,* a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae. Genes Dev.* **7,** 1160–1175.

Schwob, E., Bohm, T., Mendenhall, M. D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40*SIC1* controls the G_1 to S transition in *S. cerevisiae. Cell* **79,** 233–244.

Segal, M., Clarke, D. J., Maddox, P., Salmon, E. D., Bloom, K., and Reed, S. I. (2000). Coordinated spindle assembly, and orientation requires Clb5p-dependent kinase in budding yeast. *J. Cell Biol.* **148,** 441–452.

Seki, T., and Diffley, J. F. (2000). Stepwise assembly of initiation proteins at budding yeast replication origins *in vitro. Proc. Natl. Acad. Sci. USA* **97,** 14115–14120.

Shaikh, T. H., Gottlieb, S., Sellinger, B., Chen, F., Roe, B. A., Oakey, R. J., Emanuel, B. S., and Budarf, M. L. (1999). Characterization of *CDC45L:* A gene in the 22q11.2 deletion region expressed during murine and human development. *Mammalian Genome* **10,** 322–326.

Sherman, D. A., and Forsburg, S. L. (1998). *Schizosaccharomyces pombe* Mcm3p, an essential nuclear protein, associates tightly with Nda4p (Mcm5p). *Nucleic Acids Res.* **26,** 3955–3960.

Shirahige, K., Hori, Y., Shiraishi, K., Yamashita, M., Takahashi, K., Obuse, C., Tsurimoto, T., and Yoshikawa, H. (1998). Regulation of DNA-replication origins during cell-cycle progression. *Nature* **395,** 618–621.

Shirayama, M., Toth, A., Galova, M., and Nasmyth, K. (1999). APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature* **402,** 203–207.

Shore, D., and Nasmyth, K. (1987). Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. *Cell* **51,** 721–732.

Shuster, E. O., and Byers, B. (1989). Pachytene arrest and other meiotic effects of the start mutations in *Saccharomyces cerevisiae. Genetics* **123,** 29–43.

Sidorova, J., and Breeden, L. (1993). Analysis of the SWI4/SWI6 protein complex, which directs G_1/S-specific transcription in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **13,** 1069–1077.

Singer, J. D., Manning, B. M., and Formosa, T. (1996). Coordinating DNA replication to produce one copy of the genome requires genes that act in ubiquitin metabolism. *Mol. Cell. Biol.* **16,** 1356–1366.

Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin–ligase complex. *Cell* **91,** 209–219.

Solomon, N. A., Wright, M. B., Chang, S., Buckley, A. M., Dumas, L. B., and Gaber, R. F. (1992). Genetic and molecular analysis of *DNA43* and *DNA52:* Two new cell-cycle genes in *Saccharomyces cerevisiae. yeast* **8,** 273–289.

Sorger, P. K., and Murray, A. W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34*cdc28. Nature* **355,** 365–368.

Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., Brown, P. O., Botstein, D., and Futcher, B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* **9,** 3273–3297.

Springer, J., Nanda, I., Hoehn, K., Schmid, M., and Grummt, F. (1999). Identification and chromosomal localization of murine *ORC3,* a new member of the mouse origin recognition complex. *Cytogenet. Cell Genet.* **87,** 245–251.

Stern, B., and Nurse, P. (1996). A quantitative model for the *cdc2* control of S phase and mitosis in fission yeast. *Trends Genet.* **12,** 345–350.

Stillman, B. (1996). Cell cycle control of DNA replication. *Science* **274,** 1659–1664.

Stinchcomb, D. T., Struhl, K., and Davis, R. W. (1979). Isolation and characterisation of a yeast chromosomal replicator. *Nature* **282,** 39–43.

Story, R. M., and Steitz, T. A. (1992). Structure of the recA protein–ADP complex. *Nature* **355,** 374–376.

Strausfeld, U. P., Howell, M., Descombes, P., Chevalier, S., Rempel, R. E., Adamczewski, J., Maller, J. L., Hunt, T., and Blow, J. J. (1996). Both cyclin A and cyclin E have S-phase promoting (SPF) activity in *Xenopus* egg extracts. *J. Cell Sci.* **109,** 1555–1563.

Struhl, K., Stinchcomb, D. T., Scherer, S., and Davis, R. W. (1979). High-frequency transformation of yeast: Autonomous replication of hybrid DNA molecules. *Proc. Natl. Acad. Sci. USA* **76,** 1035–1039.

Stuart, D., and Wittenberg, C. (1994). Cell cycle-dependent transcription of *CLN2* is conferred by multiple distinct cis-acting regulatory elements *Mol. Cell. Biol.* **14,** 4788–4801.

Stuart, D., and Wittenberg, C. (1995). *CLN3,* not positive feedback, determines the timing of *CLN2* transcription in cycling cells. *Genes Dev.* **9,** 2780–2794.

Su, T. T., Yakubovich, N., and O'Farrell, P. H. (1997). Cloning of *Drosophila* MCM homologs and analysis of their requirement during embryogenesis. *Gene* **192,** 283–289.

Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A. B., and Nasmyth, K. (1991). The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae. Cell* **65,** 145–161.

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* **12,** 1969–1978.

Tada, S., Chong, J. P., Mahbubani, H. M., and Blow, J. J. (1999). The RLF-B component of the replication licensing system is distinct from Cdc6 and functions after Cdc6 binds to chromatin. *Curr. Biol.* **9,** 211–214.

Takahara, K., Bong, M., Brevard, R., Eddy, R. L., Haley, L. L., Sait, S. J., Shows, T. B., Hoffman, G. G., and Greenspan, D. S. (1996). Mouse and human homologues of the yeast origin of replication

recognition complex subunit ORC2 and chromosomal localization of the cognate human gene *ORC2L. Genomics* **31,** 119–122.

Takahashi, K., Yamada, H., and Yanagida, M. (1994). Fission yeast minichromosome loss mutants *mis* cause lethal aneuploidy and replication abnormality. *Mol. Biol. Cell* **5,** 1145–1158.

Takeda, T., Ogino, K., Matsui, E., Cho, M. K., Kumagai, H., Miyake, T., Arai, K., and Masai, H. (1999). A fission yeast gene, *him1*(+)/*dfp1*(+), encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. *Mol. Cell. Biol.* **19,** 5535–5547.

Takizawa, N., Kimura, H., and Sugimoto, K. (1995). Sequence of mouse *CDC47,* a member of the minichromosome maintenance (Mcm) family involved in the DNA replication licensing system. *Gene* **167,** 343–344.

Tanaka, T., and Nasmyth, K. (1998). Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *EMBO J.* **17,** 5182–5191.

Tanaka, T., Knapp, D., and Nasmyth, K. (1997). Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90,** 649–660.

Theis, J. F., and Newlon, C. S. (1994). Domain B of *ARS307* contains two functional elements and contributes to chromosomal replication origin function. *Mol. Cell. Biol.* **14,** 7652–7659.

Thomas, C. M., Cross, M. A., Hussain, A. A., and Smith, C. A. (1984). Analysis of copy number control elements in the region of the vegetative replication origin of the broad host range plasmid RK2. *EMBO J.* **3,** 57–63.

Thommes, P., Fett, R., Schray, B., Burkhart, R., Barnes, M., Kennedy, C., Brown, N. C., and Knippers, R. (1992). Properties of the nuclear P1 protein, a mammalian homologue of the yeast Mcm3 replication protein. *Nucleic Acids Res.* **20,** 1069–1074.

Thommes, P., Kubota, Y., Takisawa, H., and Blow, J. J. (1997). The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. *EMBO J.* **16,** 3312–3319.

Thuret, J. Y., Valay, J. G., Faye, G., and Mann, C. (1996). Civ1 (CAK *in vivo*), a novel Cdk-activating kinase. *Cell* **86,** 565–576.

Todorov, I. T., Pepperkok, R., Philipova, R. N., Kearsey, S. E., Ansorge, W., and Werner, D. (1994). A human nuclear protein with sequence homology to a family of early S phase proteins is required for entry into S phase and for cell division. *J. Cell Sci.* **107,** 253–265.

Toone, W. M., Aerne, B. L., Morgan, B. A., and Johnston, L. H. (1997). Getting started: Regulating the initiation of DNA replication in yeast. *Annu. Rev. Microbiol.* **51,** 125–149.

Toyn, J. H., Toone, W. M., Morgan, B. A., and Johnston, L. H. (1995). The activation of DNA replication in yeast. *Trends Biochem. Sci.* **20,** 70–73.

Treisman, J. E., Follette, P. J., O'Farrell, P. H., and Rubin, G. M. (1995). Cell proliferation and DNA replication defects in a *Drosophila MCM2* mutant. *Genes Dev.* **9,** 1709–1715.

Tschumper, G., and Carbon, J. (1980). Sequence of a yeast DNA fragment containing a chromosomal replicator and the *TRP1* gene. *Gene* **10,** 157–166.

Tugal, T., Zou-Yang, X. H., Gavin, K., Pappin, D., Canas, B., Kobayashi, R., Hunt, T., and Stillman, B. (1998). The Orc4p and Orc5p subunits of the *Xenopus* and human origin recognition complex are related to Orc1p and Cdc6p. *J. Biol. Chem.* **273,** 32421–32429.

Tye, B. K. (1999). MCM proteins in DNA replication. *Annu. Rev. Biochem.* **68,** 649–686.

Tye, B. K., and Sawyer, S. (2000). The hexameric eukaryotic MCM helicase: Building symmetry from nonidentical parts. *J. Biol. Chem.* **275,** 34833–34836.

Tyers, M., and Futcher, B. (1993). Far1 and Fus3 link the mating pheromone signal transduction pathway to three G_1-phase Cdc28 kinase complexes. *Mol. Cell. Biol.* **13,** 5659–5669.

Tyers, M., and Jorgensen, P. (2000). Proteolysis and the cell cycle: With this RING I do thee destroy. *Curr. Opin. Genet. Dev.* **10,** 54–64.

Tyers, M., Tokiwa, G., and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G_1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.* **12,** 1955–1968.

Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992). The Cln3–Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. *EMBO J.* **11,** 1773–1784.

Van Houten, J. V., and Newlon, C. S. (1990). Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. *Mol. Cell. Biol.* **10,** 3917–3925.

Verma, R., Annan, R. S., Huddleston, M. J., Carr, S. A., Reynard, G., and Deshaies, R. J. (1997). Phosphorylation of Sic1p by G_1 Cdk required for its degradation and entry into S phase. *Science* **278,** 455–460.

Visintin, R., Prinz, S., and Amon, A. (1997). *CDC20* and *CDH1:* A family of substrate-specific activators of APC-dependent proteolysis. *Science* **278,** 460–463.

Visintin, R., Craig, K., Hwang, E. S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell* **2,** 709–718.

Walker, J. E., Eberle, A., Gay, N. J., Runswick, M. J., and Saraste, M. (1982). Conservation of structure in proton-translocating ATPases of *Escherichia coli* and mitochondria. *Biochem. Soc. Trans.* **10,** 203–206.

Walter, J., Sun, L., and Newport, J. (1998). Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell* **1,** 519–529.

Weinreich, M., Liang, C., and Stillman, B. (1999). The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. *Proc. Natl. Acad. Sci. USA* **96,** 441–446.

Whitebread, L. A., and Dalton, S. (1995). Cdc54 belongs to the Cdc46/Mcm3 family of proteins which are essential for initiation of eukaryotic DNA replication. *Gene* **155,** 113–117.

Williams, R. S., Shohet, R. V., and Stillman, B. (1997). A human protein related to yeast Cdc6p. *Proc. Natl. Acad. Sci. USA* **94,** 142–147.

Wittenberg, C., and Reed, S. I. (1988). Control of the yeast cell cycle is associated with assembly/disassembly of the Cdc28 protein kinase complex. *Cell* **54,** 1061–1072.

Wittenberg, C., Sugimoto, K., and Reed, S. I. (1990). G_1-specific cyclins of *S. cerevisiae:* Cell cycle periodicity, regulation by mating pheromone, and association with the p34*CDC28* protein kinase. *Cell* **62,** 225–237.

Wohlgemuth, J. G., Bulboaca, G. H., Moghadam, M., Caddle, M. S., and Calos, M. P. (1994). Physical mapping of origins of replication in the fission yeast *Schizosaccharomyces pombe. Mol. Biol. Cell* **5,** 839–849.

Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to cdt1. *Science* **290,** 2309–2312.

Wold, M. S. (1997). Replication protein A: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem.* **66,** 61–92.

Wynne, J., and Treisman, R. (1992). SRF and MCM1 have related but distinct DNA binding specificities. *Nucleic Acids Res.* **20,** 3297–3303.

Yamashita, M., Hori, Y., Shinomiya, T., Obuse, C., Tsurimoto, T., Yoshikawa, H., and Shirahige, K. (1997). The efficiency and timing of initiation of replication of multiple replicons of *Saccharomyces cerevisiae* chromosome VI. *Genes Cells* **2,** 655–665.

Yan, H., Gibson, S., and Tye, B. K. (1991). Mcm2 and Mcm3, two proteins important for *ARS* activity, are related in structure and function. *Genes Dev.* **5,** 944–957.

Yan, H., Merchant, A. M., and Tye, B. K. (1993). Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. *Genes Dev.* **7,** 2149–2160.

Yeong, F. M., Lim, H. H., Padmashree, C. G., and Surana, U. (2000). Exit from mitosis in budding yeast: Biphasic inactivation of the Cdc28–Clb2 mitotic kinase and the role of Cdc20. *Mol. Cell* **5,** 501–511.

Yoon, H. J., and Campbell, J. L. (1991). The CDC7 protein of *Saccharomyces cerevisia*e is a phosphoprotein that contains protein kinase activity. *Proc. Natl. Acad. Sci. USA* **88,** 3574–3578.

Yoon, H. J., Loo, S., and Campbell, J. L. (1993). Regulation of *Saccharomyces cerevisiae* CDC7 function during the cell cycle. *Mol. Biol. Cell* **4,** 195–208.

You, Z., Komamura, Y., and Ishimi, Y. (1999). Biochemical analysis of the intrinsic Mcm4–Mcm6–mcm7 DNA helicase activity. *Mol. Cell. Biol.* **19,** 8003–8015.

Young, M. R., and Tye, B. K. (1997). Mcm2 and Mcm3 are constitutive nuclear proteins that exhibit distinct isoforms and bind chromatin during specific cell cycle stages of *Saccharomyces cerevisiae. Mol. Biol. Cell* **8,** 1587–1601.

Young, M. R., Suzuki, K., Yan, H., Gibson, S., and Tye, B. K. (1997). Nuclear accumulation of *Saccharomyces cerevisiae* Mcm3 is dependent on its nuclear localization sequence. *Genes Cells* **2,** 631–643.

Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: Cell division and the anaphase-promoting complex. *Genes Dev.* **13,** 2039–2058.

Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. (1998). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* **282,** 1721–1724.

Zhou, C., and Jong, A. (1990). *CDC6* mRNA fluctuates periodically in the yeast cell cycle. *J. Biol. Chem.* **265,** 19904–19909.

Zisimopoulou, P., Staib, C., Nanda, I., Schmid, M., and Grummt, F. (1998). Mouse homolog of the yeast origin recognition complex subunit ORC1 and chromosomal localization of the cognate mouse gene *Orc1*. *Mol. Gen. Genet.* **260,** 295–299.

Zou, L., and Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science* **280,** 593–596.

Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p–Dbf4p kinase. *Mol. Cell. Biol.* **20,** 3086–3096.

Zou, L., Mitchell, J., and Stillman, B. (1997). *CDC45,* a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. *Mol. Cell. Biol.* **17,** 553–563.

Zwerschke, W., Rottjakob, H. W., and Kuntzel, H. (1994). The *Saccharomyces cerevisiae CDC6* gene is transcribed at late mitosis and encodes a ATP/GTPase controlling S phase initiation. *J. Biol. Chem.* **269,** 23351–23356.

This Page Intentionally Left Blank

Phenotype Switching in Polymorphic *Tetrahymena:* A Single-Cell Jekyll and Hyde

Phillip E. Ryals,* Harriett E. Smith-Somerville,† and Howard E. Buhse, Jr.‡
*Department of Biochemistry and Molecular Biology, Mississippi State University, Mississippi State, Mississippi 39762; †Department of Biological Sciences and Coalition for Biomolecular Products, University of Alabama, Tuscaloosa, Alabama 35487; and ‡Department of Biological Sciences, University of Illinois, Chicago, Illinois 60607

For nearly half a century, phenotype switching in the group of polymorphic species of the ciliate genus *Tetrahymena* has been the subject of investigations of the underlying mechanisms, the accompanying biochemical and structural changes, and the evolution of polymorphic survival strategy. Beginning with the pioneering systematic studies by Furgason in 1940 of hymenostome ciliates, the experimental approach rapidly expanded to include investigations of growth, nutrition, physiology, morphology, and morphogenesis in the polymorphic species. Recently, with progress in elucidation of the novel signaling ligand and identification of elements of the subsequent signal transduction cascade, in addition to the growing catalog of intracellular events associated with differentiation in these unicellular eukaryotes, we have begun to address the mechanistic basis of polymorphism. This review summarizes and integrates the history and recent discoveries concerning *Tetrahymena* polymorphic cells. We are now poised to answer fundamental questions about this interesting pathway of cell differentiation.

KEY WORDS: Cell signaling, Ciliate polymorphism, Differentiation, Protozoa, *Tetrahymena.*

I. Introduction

Ciliates are bad and good for cell biologists. Bad because they undermine our self-esteem by showing how little we understand cell structure at the molecular level;

0074-7696/02 $35.00

> good because they offer us problems, which, when solved, will be a quantal leap in our understanding of not only cells but, surprisingly, embryos.
>
> — Wolpert (1990) in his review of the book *Pattern Formation: Ciliate Studies and Models* by Frankel (1989)

Among the properties shared by certain protozoa and embryonic cells is the ability to undergo differentiation involving changes in form and/or function (Trager, 1963). Included in these protozoa are six described species of the unicellular eukaryote *Tetrahymena* that can dramatically alter their morphology in response to defined changes in the extracellular environment. This inducible alteration, often referred to as transformation, occurs in *T. vorax, T. patula, T. leucophrys* (formerly *T. vorax* Tur), *T. caudata, T. paravorax,* and *T. silvana* (Corliss, 1953, 1957; Dragesco and Njiné, 1971; Elliott and Hayes, 1954; Kidder *et al.,* 1940; Njiné, 1972; Simon *et al.,* 1985; Williams, 1960, 1961; Williams *et al.,* 1984). These ciliates exist as one of two alternative trophic forms—microstomal or macrostomal. The microstomal form feeds by filtering particulate material including microorganisms. The microstomal cell differentiates into the macrostomal form under low-nutrient conditions in the presence of a stimulus, which may be supplied by the prey (Buhse, 1966a, 1967a; Claff, 1947). This cell is a carnivore capable of ingesting prey ciliates. Consistent with the divergent food supply of the two cell types, the major morphological differences reside in the cytoskeletal and membrane-limited feeding structures. A third phenotype (not discussed further), the reproductive cyst, has been described in several of the species (Gabe and Williams, 1982; Simon *et al.,* 1985; Williams, 1960, 1961). The transition among different cell types is representative of a "simple" form of nonterminal differentiation. By understanding differentiation in such a system in which the component events can be controlled to a large degree, better insight into differentiation in more complex systems can be gained.

Of the species capable of undergoing differentiation, *T. vorax* Kidder, 1941 [also known as *Glaucoma vorax* or *Leucophrys vorax* in the early literature (Corliss, 1953, 1973)] is the most extensively studied. Comparative analyses of the microstomal and macrostomal cell types have detailed the qualitative and quantitative differences in cellular phenotype. Recent studies have provided information on the chemical nature of the naturally occurring signal responsible for triggering the differentiation process and have begun to explore the biochemical and molecular mechanisms that direct the response to the novel signal. This review was undertaken to present the current state of knowledge regarding this remarkable system emphasizing *T. vorax,* and to explore the broader implications of the findings.

II. Evolution of Polymorphism in *Tetrahymena*

Recent phylogenies for the genus *Tetrahymena* based on rRNA sequence analysis (Nanney *et al.,* 1989, 1998; Preparata *et al.,* 1989), isozyme patterns (Nanney

et al., 1989), cytoskeletal protein patterns (Williams *et al.,* 1984), and histone H3 and H4 sequence analysis (Brunk *et al.,* 1990; Sadler and Brunk, 1992) have revealed a surprising aspect of this polymorphism. The species capable of carnivory are widely, but not uniformly, scattered with respect to their phylogenetic placements. In most phylogenies, the differentiating species *T. vorax, T. leucophrys,* and *T. silvana* are grouped together in a cluster which includes several nondifferentiating species. On the other hand, the phylogenetic tree based on histone H4 sequence analysis separates *T. leucophrys* from *T. vorax* and *T. silvana*. A fourth polymorphic species, *T. patula,* is placed in a separate cluster in all phylogenies. *Tetrahymena paravorax,* the most aberrant species, and *T. caudata* are believed to be the most primitive of the tetrahymenines (Nanney *et al.,* 1989). The distribution of these species suggests either that phenotype switching is a primitive trait lost in some lineages or that it independently evolved in several lineages (Nanney *et al.,* 1989). The pseudomacrostome mutations in *T. thermophila,* a nondifferentiating species, indicate that similar mutations could have occurred more than once as an initial step in the evolution of differentiation (Frankel, 1983).

III. Polymorphic *Tetrahymena* as Experimental Organisms

The extant strains of the polymorphic species held by the American Type Culture Collection are listed in Table I. A distinct advantage of these cells is the short generation time (6 or 7 h in *T. vorax*), which is conducive to rapidly producing cultures of high cell density for experimental studies. Clonally pure cell lines can

TABLE I
Strains of Polymorphic *Tetrahymena* Available through the American Type Culture Collection

Species	Strain	Micronucleus	Origin and date	Reference
T. caudata	MP49	Micronucleate	Jungle stream, Malaysia, 1980	Simon *et al.* (1985)
	MP107	Micronucleate	Pond, University Pertanian, Malaysia, 1980	Simon *et al.* (1985)
T. leucophrys	Tur	Amicronucleate	Turtox Supply Company Culture	Whitson (1961)
T. paravorax	RP	Micronucleate	Republic of Panama, 1954	Elliott and Hayes (1954)
T. patula	LI	Micronucleate	Freshwater pond, Port Washington, NY, 1950	Corliss (1953)
	L-FF	Amicronucleate	Paris, France, 1948	Corliss (1953)
T. silvana	MP67	Micronucleate	Jungle stream, University Pertanian, Malaysia, 1980	Simon *et al.* (1985)
	MP68	Micronucleate	Jungle stream, University Pertanian, Malaysia, 1980	Simon *et al.* (1985)
T. vorax	V_2S	Amicronucleate	Pond, Rhode Island, 1940	Corliss (1953)

be generated. Many results from biochemical and molecular studies, including stages of differentiation, can be correlated with readily identifiable morphological markers. Cell division and differentiation are easily synchronized by a variety of methods (Buhse, 1966a,b, 1967a; Buhse and Rasmussen, 1974; Stone, 1963; Williams, 1964). This simple cell possesses many shared biochemical and molecular pathways, permitting extrapolation of results to higher eukaryotes.

A. Nutritional Requirements

Cultures of *Tetrahymena* polymorphs can be grown axenically in both complex and chemically defined media. Rapid cell division and high-density cultures are obtained in a simple medium composed of 2% proteose peptone, 0.5% glucose, and 0.2% yeast extract, supplemented with an Fe^{2+}–EDTA complex (Nelsen *et al.,* 1981; Thompson, 1967). Other media commonly employed in the culture of *Tetrahymena* include Loefer's medium (Loefer *et al.,* 1958) and the synthetic media of Kidder and Dewey (1951), Holz *et al.* (1962), and Szablewski *et al.* (1991).

The nutritional requirements for optimum growth of four of the six species of polymorphic *Tetrahymena* in a chemically defined medium are summarized in Table II. There is no information on the requirements of *T. caudata* or *T. silvana*. The polymorphic species share an absolute dietary requirement for a purine and pyrimidine with other members of the genus, a result of the inability of these cells to synthesize either the purine or pyrimidine ring (Conner and Koroly, 1973; Holz, 1973). Asolectin, a soybean extract containing phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, plus small amounts of other polar lipids, will support good growth in chemically defined medium (Luksas and Erwin, 1983; Shaw and Williams, 1963). Both *T. patula* and *T. paravorax* can be grown in media containing certain sterols in place of fatty acid-containing lipids, although growth of *T. patula* is suboptimal (Holz *et al.,* 1961; Luksas and Erwin, 1983). The metabolic basis for the nutritional equivalence of sterols and fatty acid-containing lipids is unknown.

Tetrahymena vorax can be cloned readily in both complex and synthetic media (Schousboe *et al.,* 1992). Cloning efficiency for cells transferred from a complex medium into a chemically-defined medium is increased when the synthetic medium is augmented with hemin.

B. Culture Conditions

Some debate exists regarding optimum surface area to volume ratios, optimum growth temperature, and the benefits of still versus shaken culture as these factors relate to the differentiation process. In general, for *T. vorax,* high surface area to volume ratios are preferred when maintaining still cultures (Buhse, 1966a). Cultures of larger volume benefit from moderate reciprocal shaking at approximately 90 rpm.

TABLE II
Nutritional Requirements for Optimum Growth of Polymorphic *Tetrahymena* in Chemically Defined Medium[a]

Nutrient	*T. leucophrys* Tur	*T. paravorax* RP	*T. patula* LI	*T. patula* L-FF	*T. vorax* V_2
Amino Acid					
L-Arginine	+	+	+	+	+
Glycine	−	+	+	−	−
L-Histidine	+	+	+	+	+
L-Isoleucine	+	+	+	+	+
L-Leucine	+	+	+	+	+
L-Lysine	+	+	+	+	+
L-Methionine	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+
L-Serine	+	+	+	+	+
L-Threonine	+	+	+	+	+
L-Tryptophan	+	+	+	+	+
L-Valine	+	+	+	+	+
Vitamin					
Nicotinic acid	+	+	+	+	+
Thiamine	+	+	+	+	+
Riboflavin	+	+	+	+	+
Pantothenic acid	+	+	+	+	+
Folic acid	+	+	+	+	+
Thioctic acid	+	+	+	+	+
B_6	+	−	+	+	+
Purine/pyrimidine					
Guanine or derivative	+	+	+	+	+
Uracil, cytidine, or derivative	+	+	+	+	+
Lipid					
Sterol	Not tested	+	Not tested	−	Not tested
Phospholipids	+	+[b]	+	+	+
Carbohydrate					
Glucose or dextrin	+	+	+	+	+

[a] Compiled from Holz (1964, 1973), Holz *et al.* (1961), Lascelles (1957), and Shaw and Williams (1963).
[b] This species can utilize either sterols or phospholipids for optimum growth. The sterol requirement can be eliminated by inclusion of combinations of synthetic phosphoglycerides, oleic acid, and glycerophosphate in the medium (Holz, 1973).

The growth temperature employed in many investigations is 20°C; however, incubation at 24 or 25°C stimulates more rapid cell division and does not appear to be detrimental to cells or to their ability to differentiate. Temperatures in excess of ~30°C are lethal to *T. vorax* (Buhse and Rasmussen, 1974). Growth of *T. patula* at 25°C instead of 20°C reduced the generation time from 8 to 6.5 h; however, gradual adaptation of the cells to the higher temperature was required to achieve this more rapid growth (Stone, 1963).

Tetrahymena vorax can be maintained for 2 weeks by inoculating 1 or 2 ml of 2% proteose peptone in culture tubes with several drops of culture from a Pasteur pipet (P. Ryals, unpublished observation). Anaerobic backstocks prepared using the method described by Williams *et al.* (1980) contain viable cells for approximately 6 months. The cells can be backstocked by cryopreservation at liquid nitrogen temperatures following the procedure described by Cassidy-Hanley *et al.* (1995), and active cultures can be restored after 1 year or longer.

IV. Characteristics of *Tetrahymena* Microstomal and Macrostomal Cell Types

A. General Structural Characteristics

The overall appearance and dimensions of the *Tetrahymena* microstomal and macrostomal phenotypes are markedly different from each other even at the gross morphological level (Fig. 1). At the ultrastructural level, the two cell types have been shown to differ in a more pronounced fashion, particularly in the cytoskeletal organization of the oral apparatus and in vacuole characteristics. The two phenotypes of *T. vorax* are compared in Table III.

Each cell type possesses the usual array of eukaryotic cellular organelles characteristic of *Tetrahymena* (Elliott and Kennedy, 1973). All *Tetrahymena* are bounded

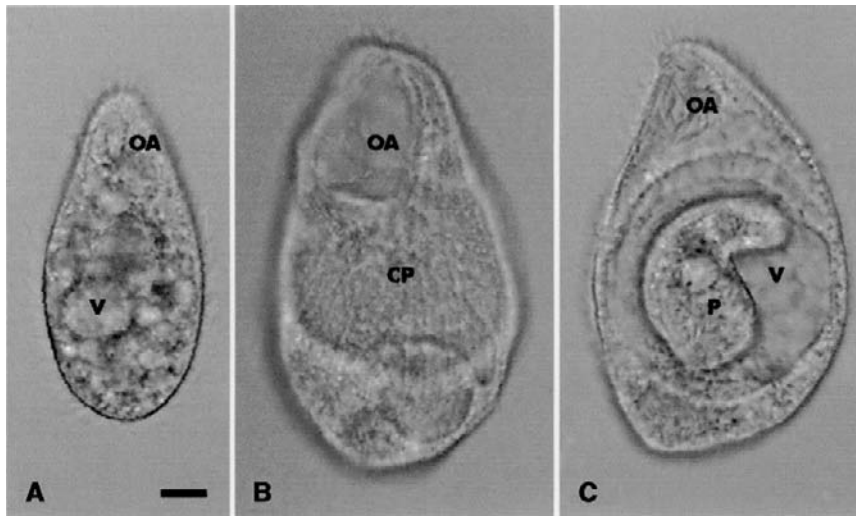


FIG. 1 Cell types of *T. vorax.* (A) Microstomal cell with a small oral apparatus (OA) and numerous vacuoles (V). (B) Macrostomal cell with large OA and cytopharyngeal pouch (CP). (C) Macrostomal cell with a sealed vacuole (V) containing a recently ingested prey ciliate (P). Scale bar = 10 μm.

TABLE III

Comparison of Major Phenotypic Differences between Microstomal and Macrostomal Cells of *Tetrahymena vorax*

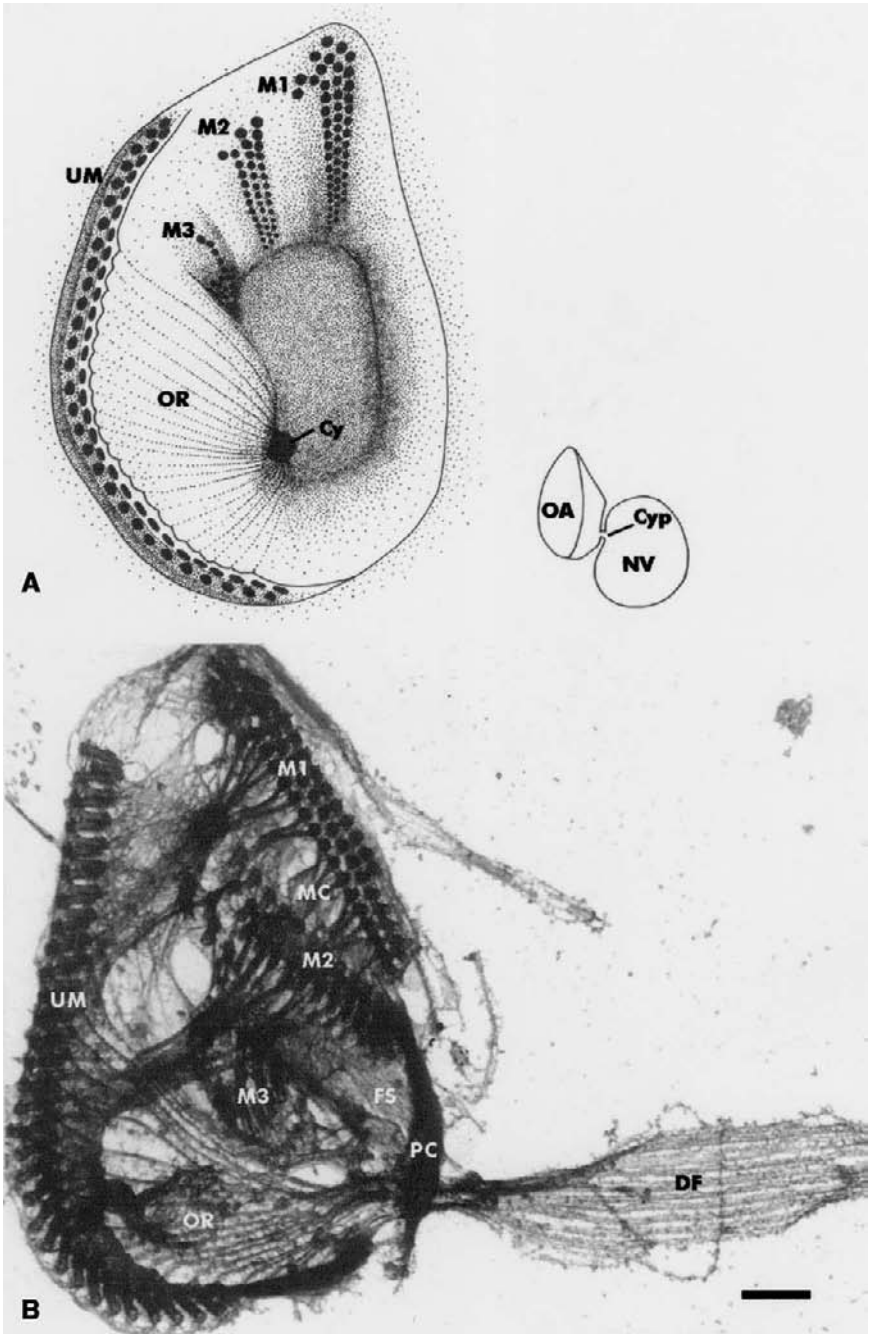
Characteristic	Microstomal cell	Macrostomal cell	Reference
Cell			
Cell shape	Prolate spheroid	"Rounded" prolate spheroid	
Cell length (μm, mean)	77.7	110	Williams (1961)
Cell width (μm, mean)	29.2	75	Williams (1961)
Cell surface area (μm^2, mean)	6554	11,689	Smith-Somerville *et al.* (1986)
Oral apparatus			Smith (1982a,b)
Length (μm, mean)	10.6	29	
Width (μm, mean)	5.7	23	
Arrangement of membranelle kinetosomes			
Membranelle 1	3 rows	3 rows	
Membranelle 2	3 rows	3 rows	
Membranelle 3	J-shaped	3 rows	
Number of kinetosomes in membranelle 1			
Row 1	12–13	17–22	
Rows 2 and 3	19–20	27–29	
Number of kinetosomes in membranelle 2			
Row 1	14–15	18–23	
Rows 2 and 3	14–15	24–28	
Number of kinetosomes in membranelle 3	15–18		
Row 1	J-shaped, no rows	12–18	
Rows 2 and 3		18–25	
Kinetosomes in undulating membrane			
Arrangement	1 ciliated and 1 nonciliated row	1 ciliated and 1 nonciliated row	
Number	28–30 in each row	72–82 in each row	
Number of oral connectives between membranelles and undulating membrane	4	1	
Origin of first oral rib	Seventh kinetosome of undulating membrane	First kinetosome of undulating membrane	
Diameter of cytostome (μm)	0.15–0.20	15–16	
Vacuoles			Smith-Somerville *et al.* (1986)
Vacuoles/cell	33.3	6.6 + cytopharyngeal pouch	
Total vacuole surface area	5310 μm^2	6953 μm^2 total and 5715 μm^2 pouch only	

by a pellicle consisting of a triple membrane system and the epiplasm, which lies beneath the membranes (Allen, 1967; Pitelka, 1961). The plasma membrane, which covers the body and cilia, plus the outer and inner alveolar membranes constitute the three membranes. The plasma and outer alveolar membranes are separated by a constant distance of 1.2–1.25 nm connected by struts at 9-nm intervals (Allen, 1967; Satir and Wissig, 1982; Tokuyasu and Scherbaum, 1965). The outer and inner alveolar membranes are continuous around the base of the cilia along the primary meridians and at the sites for mucocyst fusion along the primary and secondary meridians (Allen, 1967). This creates a closed alveolus or alveolar sac. Parasomal sacs, which structurally resemble caveolae in mammalian cells (Anderson *et al.,* 1992), occur in the plasma membrane anterior to each somatic cilium. These sacs are believed to be sites of endocytosis. The membrane of the parasomal sacs has coated regions, and cationized ferritin, a nonspecific polyvalent tracer for endocytosis, labels both coated pits and internal coated vesicles (Allen, 1967; Mislan and Smith-Somerville, 1986; Nilsson and van Deurs, 1983). The pellicle is supported by arrays of longitudinal, transverse, and postciliary microtubules (Allen, 1967). The epiplasm or membrane skeleton, basal bodies (kinetosomes) and associated kinetodesmal fibers, and microtubule arrays remain in the insoluble residue when *Tetrahymena* is extracted with Triton X-100 (Collins *et al.,* 1980; Vaudaux, 1976; Williams *et al.,* 1979). Buhse and Williams (1982) found no differences in the protein composition of this insoluble cytoskeleton between microstomal and macrostomal cells of *T. vorax* in one-dimensional electrophoretic gels.

Although mitochondria are located throughout the cell, many are localized along the microtubule arrays at the periphery (Aufderheide, 1979). In *Tetrahymena,* Golgi bodies composed of one or two cisternae are also concentrated in the cell cortex, often closely associated with the mitochondria (Kurz and Tiedtke, 1993). Mucocysts, small membrane-bound organelles with ordered crystalline interiors, are found in the cytoplasm adjacent to the plasma membrane (Allen, 1967; Tokuyasu and Scherbaum, 1965). These structures fuse with the pellicle and can be induced to discharge their contents in response to mechanical or chemical stimuli, low pH, or osmotic shock (Nilsson, 1979; Suhr-Jessen, 1987; Thompson *et al.,* 1974; Tiedtke *et al.,* 1988). The function of mucocysts is unknown, although the mucous has been suggested to concentrate solutes and ions from the medium and to assist in the uptake of these absorbed solutes during feeding (Nilsson, 1979).

B. The Oral Apparatus

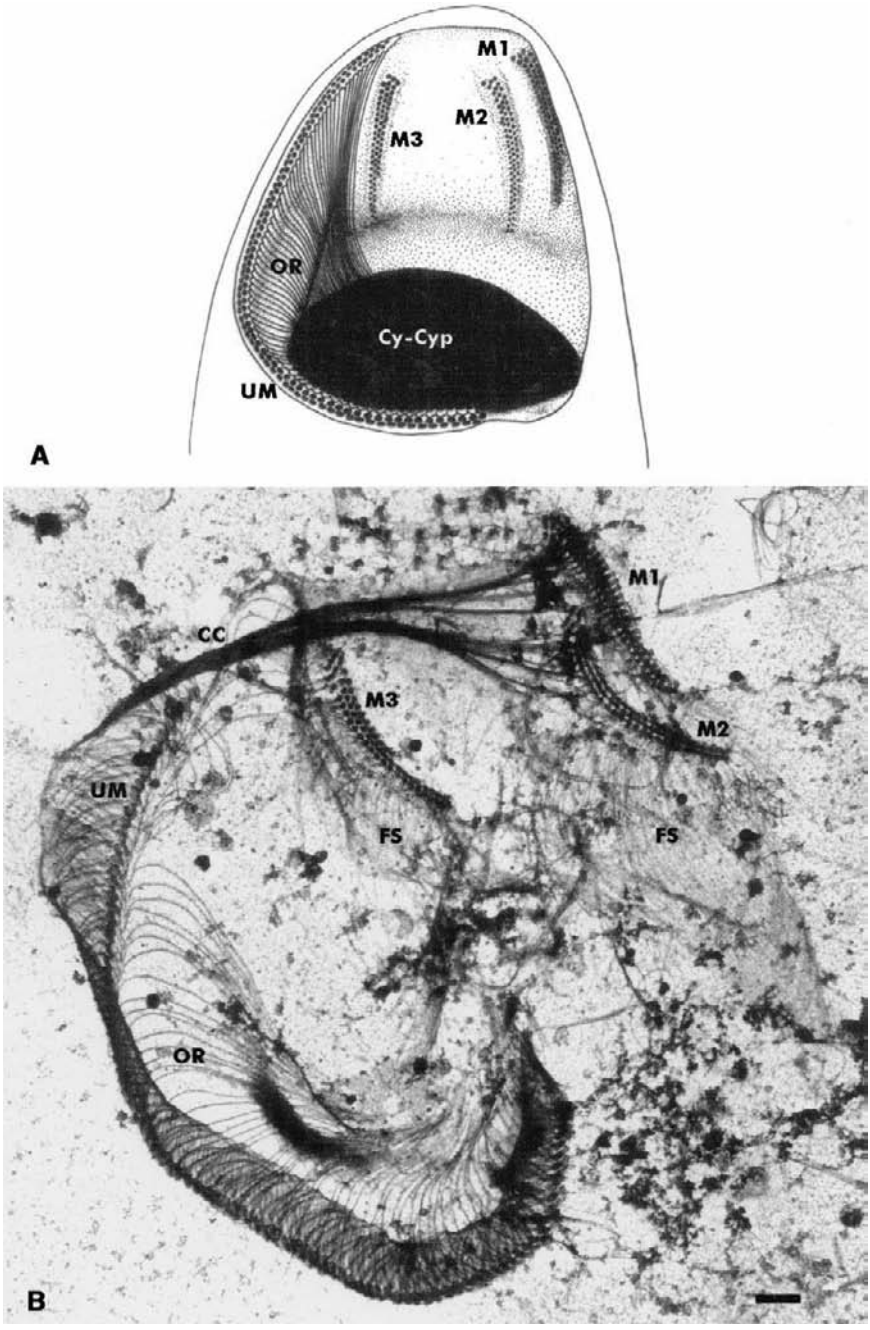
In *Tetrahymena,* the oral apparatus is located on the anterior ventral surface. The structure in the microstomal cell is typical for the genus (Smith, 1982a; Smith and Buhse, 1983). The oral apparatus includes a buccal cavity bordered on the right side by a ciliary undulating membrane, three membranelles on the anterior wall, and a complex cytoskeleton (Fig. 2). The membranelles are connected to


M1
M2
UM
M3
OR
Cy
OA
Cyp
NV
A
M1
MC
M2
UM
M3
FS
PC
DF
OR
B

each other by membranellar connectives composed of rows of microtubules that continue under the pellicular membrane of the left and dorsal walls as the fibrillar sheet. Additional microtubules form the oral connectives joining the membranelles with the undulating membrane and support the oral ribs along the right and posterior walls. Part of the oral rib microtubules extends past the oral cavity as the deep fiber (Smith and Buhse, 1983). Four filamentous structures contribute to the cytoskeleton: (i) the pellicular or terminal plates to which the basal bodies of the membranelles are connected, (ii) the coarse filamentous reticulum which extends into the cytoplasm below the undulating membrane, (iii) the fine filamentous reticulum beneath the oral ribs, and (iv) the filamentous specialized cytoplasm which lines the left and dorsal walls of the posterior portion of the oral apparatus and embeds the microtubules of the deep fiber. Microstomal cells collect food from the surrounding environment by using the ciliature to sweep particles and concentrate them in small phagosomes attached to the cytostome–cytopharyngeal complex. This complex is located in the posterior dorsal wall of the buccal cavity and consists of the aperture and channel connecting the buccal cavity with the vacuole.

The oral apparatus of the macrostomal cell has the same basic structural features but exhibits quantitative and qualitative differences (Miller and Stone, 1963; Smith, 1982b; Smith-Somerville and Buhse, 1984). This structure is larger than that of the microstomal form, and it contains an enlarged cytostome–cytopharyngeal complex that occupies most of the posterior part of the buccal cavity (Fig. 3A). Like that of the microstome, the oral apparatus has three ciliary membranelles and an undulating membrane, but those in the macrostome are longer (Fig. 3B). There are also differences in the arrangement of basal bodies and in membranelle placement. In the microstomal oral apparatus, the membranelles are joined to the undulating membrane by four connectives. In contrast, the oral cytoskeleton of the macrostomal cell has only one cross-connective which joins the anterior ends of the three membranelles to the anterior end of the undulating membrane. Additional differences include the number of oral ribs, origin of the first oral rib, and the

FIG. 2 Oral apparatus of the microstomal cell type of *T. vorax* viewed from the direction of the ventral surface. (A) Three-dimensional drawings illustrating the general shape of the oral cavity and position of the cytostome (Cy) near the posterior end. Front view shows the three ciliary membranelles (M1–M3), the undulating membrane (UM) with one ciliated and one nonciliated row of kinetosomes, and oral ribs (OR) lining the (organism's) right side of the buccal cavity. The first oral rib originates at the seventh pair of kinetosomes in the UM. Side view shows a nascent vacuole (NV) attached to the oral apparatus (OA) through the cytopharynx (Cyp) (reproduced with permission from Smith, 1982a, Fig. 6, p. 42). (B) Isolated oral apparatus. The OR microtubules extend from the kinetosomes of the UM past the posterior connective (PC) to form the deep fiber (DF). The membranelles are connected by membranellar connectives (MC). Membranelles 1 and 2 (M1 and M2) contain three rows of kinetosomes, whereas M3 is J-shaped. The microtubules of the fibrillar sheet (FS) line the dorsal and the organism's left walls of the oral cavity. Scale bar = 1 μm (reproduced with permission from Smith and Buhse, 1983, Fig. 2, p. 266).


M1
M2
M3
OR
Cy-Cyp
UM
A
M1
CC
M3
M2
UM
FS
FS
OR
B

presence of 55 polypeptides unique to the macrostomal oral skeleton (Gulliksen *et al.,* 1984; Smith, 1982b).

During transition into the macrostomal form, the oral apparatus of the microstomal cell is replaced by that characteristic of the macrostomal cell (Table IV, Fig. 4). Oral replacement begins with development of a small anarchic field of basal bodies on the ventral surface posterior to the microstomal oral apparatus (Buhse, 1966b; Buhse *et al.,* 1970). This field enlarges, and the membranelles become organized beginning with membranelle 1 as the original oral apparatus is resorbed. The undulating membrane and oral ribs form during later stages of stomatogenesis, ending with the "in-sinking" to create a definitive buccal cavity. This movement results in the separation of the second and third membranelles. The placement of the membranelles and the undulating membrane in the macrostomal form preclude concentration of bacteria and particles in the nascent vacuole.

Unlike differentiation into the macrostomal cell type, the transition from the macrostome to the microstome is accompanied by division of the cell (Buhse, 1966b). The appearance of a small anarchic field of basal bodies in the midregion of the macrostome marks the beginning of oral development for the opisthe (posterior daughter cell). As this oral apparatus develops, the macrostomal oral apparatus is remodeled into that characteristic of a microstomal cell for the protor (anterior daughter cell).

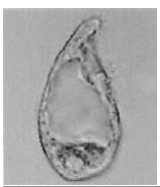
C. The Phagosomes

Phagotrophy in microstomal cells is similar to that in nondifferentiating species of *Tetrahymena*. Feeding is accomplished by uptake of particulate material through the buccal cavity and cytostome into food vacuoles (phagosomes). In the macrostomal cell, feeding is "carnivorous" and sometimes "cannibalistic," involving the trapping and digestion of other protozoa in the cytopharyngeal pouch. This form of phagocytosis occurs only in the differentiating species of *Tetrahymena*.

FIG. 3 Oral apparatus of the macrostomal cell type of *T. vorax* viewed from the direction of the ventral surface. (A) Three-dimensional drawing illustrating the general shape of the oral cavity and position of the cytostome–cytopharyngeal complex (Cy–Cyp). The undulating membrane (UM) borders the organism's right margin and part of the posterior margin of the oral cavity. Membranelle 1 (M1) is adjacent to the organism's left surface. Membranelle 2 (M2) lies next to M1. Membranelle 3 (M3) is positioned near the organism's right surface separated from M1 and M2. The right surface is lined by oral ribs (OR). The first OR originated at the most anterior pair of kinetosomes in the UM (reproduced with permission from Smith, 1982b, Fig. 5, p. 619). (B) Isolated oral apparatus. The UM that curves along the right side has folded over. Microtubules of the OR extend from the UM. The three membranelles (M1–M3) are connected to the top of the UM by a cross-connective (CC). Each membranelle contains a short row and two longer rows of kinetosomes. Fibrillar sheet microtubules (FS) extend from the membranelles. Scale bar = 2 μm (reproduced with permission from Smith, 1982b, Fig. 10, p. 621).

TABLE IV

Time Course for Stomatin-Induced Differentiation in *Tetrahymena vorax*

Characteristic phenotype[a]	Event	Fraction of differentiation time	Arrest of differentiation: Exposure to protease[b]	Arrest of differentiation: Addition of cerulenin	Arrest of differentiation: Addition of transcription inhibitors[c]	Arrest of differentiation: Addition of translation inhibitors[d]
	Induction of differentiation in late logarithmic/early stationary phase of growth	0				
		0.1				
	Initiation of increase in transcription and translation; initiation of decline in respiration	0.2				
		0.3				
	Maximum increase in transcription	0.4				
	Initiation of oral replacement					
	Initiation of increase in respiration	0.5				
	Maximum increase in translation	0.6				
		0.7				
		0.8				
	Oral replacement complete; initiation of cytopharyngeal pouch development	0.9				
	Process complete	1.0				

[a] Data compiled from Buhse (1966a,1967a,1967b), Buhse and Cameron (1968), Buhse and Hamburger (1974), Buhse *et al.* (1991), Grelland (1988b), Nicolette *et al.* (1971), and Ryals *et al.* (1999).

[b] Proteinase K, proteinase E, and trypsin (Ryals *et al.,* 1999).

[c] Actinomycin D, 5-fluoro-2-deoxyuridine, and 2-mercapto-1-(β-4-pyridethyl) benzimidazole (Buhse and Hamburger, 1974; Nicolette *et al.,* 1971).

[d] *para*-Fluorophenylalanine (Buhse, 1967b; Buhse and Hamburger, 1974).

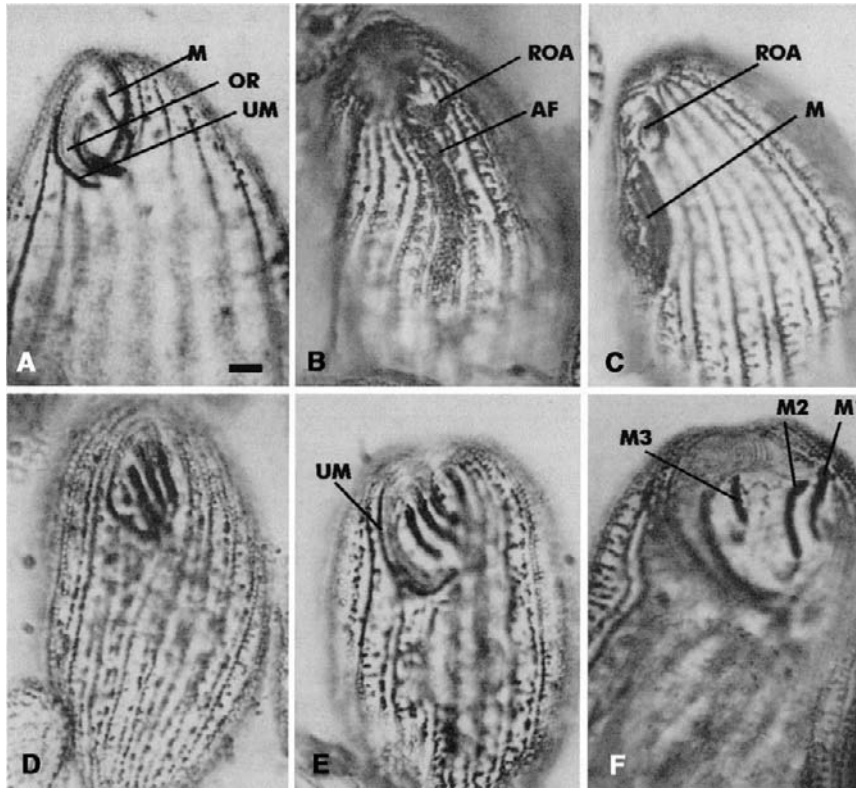


FIG. 4 Successive stages in oral replacement. Cells were prepared by the Chatton–Lwoff silver technique. (A) A representative microstome showing membranelles (M), undulating membrane (UM), and oral ribs (OR). Scale bar = 5 μm. (B) An early stage in macrostome formation. The anarchic field (AF) is forming below the resorbing oral apparatus (ROA). (C) A later stage in oral replacement. The anarchic field of kinetosomes has begun to organize into membranelles (M), while the ROA is pushed forward. (D) The old oral apparatus is completely resorbed, and the field of kinetosomes has organized into the new oral apparatus. (E) The UM has developed, and the membranelles are moving into position. (F) Completed macrostomal oral apparatus with the large buccal cavity, undulating membrane, and membranelles. Membranelle 3 (M3) is separated from M1 and M2 (reproduced with permission from Buhse, 1966b, Figs.1–6, p. 306).

In microstomal cells, phagosomes are formed sequentially. New vacuoles in undifferentiated *T. vorax* have a mean diameter of 7.5–9.6 μm depending on the method of measurement (Rasmussen *et al.,* 1975; Smith-Somerville *et al.,* 1986). When phagocytosis is stimulated by the addition of particles to the medium, vacuoles are formed at a maximum rate of 0.44 ($\pm$0.1) vacuoles/min.

Phagosome processing is similar to that described for *Paramecium* (Allen and Fok, 2000; Mislan and Smith-Somerville, 1986; Smith-Somerville, 1989; Smith-Somerville *et al.,* 1986, 1991). Studies of processing in *Tetrahymena* are facilitated

by the ability to load nascent vacuoles with iron, a material that permits separation of these phagosomes on the basis of weight or magnetic properties (Vosskühler and Tiedtke, 1993; Weidenbach and Thompson, 1974). Experiments using this methodology have revealed that vacuoles in *T. vorax* proceed through a series of highly ordered events. The nascent vacuole forms by fusion of flattened, disk-shaped cytosolic membrane vesicles at the base of the cytopharynx. Following separation, phagosomes undergo a mandatory period of processing lasting about 100 min before they become capable of fusing with the cytoproct. Acidosomes surround the vacuole and begin to fuse with it soon after separation. Acidification is complete by 30 min, the time at which minimum vacuole pH is achieved. During this time, membrane vesicles are removed from the vacuole. Acid phosphatase appears between 30 and 60 min following initiation of vacuole formation, signaling the fusion of lysosomes and the beginning of digestion. Late in the digestive period, vacuole pH rises, the level of acid phosphatase activity declines, and additional membrane vesicles are removed from the vacuole. Vacuoles that are advanced in the processing cycle fuse with the cytoproct and undigested material is discharged. *Tetrahymena* appears to lack the cytoskeletal elements found in *Paramecium* that direct vesicles retrieved from vacuoles back to the cytopharynx.

The cytopharyngeal pouch, the nascent vacuole in the macrostome, forms as part of the cellular phenotype during the last 30–60 min of differentiation by the fusion of vesicles with the base of the cytopharynx (Holsen and Buhse, 1969; Smith, 1982b). As the pouch develops, the macronucleus, which is centrally located in the microstomal cell, is displaced to the posterior end of the cell. This pouch is sufficiently large to hold a prey ciliate such as *T. pyriformis, T. thermophila, Colpidium,* or microstomal forms of *T. vorax* (Fig. 1c). The pouch can occupy as much as 80% of the cell volume and has a diameter of 35–40 μm (Smith-Somerville *et al.,* 1986) (Table III). It is open to the external environment through a large cytostome–cytopharyngeal complex which occupies most of the posterior end of the buccal cavity, until it seals to form a phagosome upon entry of a suitable prey organism. Initially, this vacuole is sufficiently large for a prey protozoan to move about fieely within it (Fig. 1c), but as the phagosome moves posteriorly it decreases in size and the prey is compressed (Sherman *et al.,* 1982). A new pouch begins to develop shortly after separation of the previous phagosome and reaches its maximum size within 30 min (Sherman *et al.,* 1981). The digestive phase appears to be much longer than that in the microstomal cell since in the presence of plentiful prey, many vacuoles containing partially digested protozoa often accumulate in the posterior area.

D. Physiological Properties of the Cytopharyngeal Pouch

The macrostomal cell type is stable for several hours in the absence of prey before it undergoes division to form two microstomal daughter cells. This period allows

ample time for experimental manipulation of the macrostomal population. Of considerable interest is the means by which the cytopharyngeal pouch is formed and maintained. Specific compounds of biological significance have been shown to affect the shape and integrity of the cytopharyngeal pouch. Puromycin (3 m*M*) and cycloheximide (5 mg/ml), both potent inhibitors of protein synthesis in *T. vorax,* induce cytopharyngeal pouch collapse when added to suspensions of macrostomal cells (Buhse *et al.,* 1978). Reconstruction of the cytopharyngeal pouch occurs when these agents are removed. Cycloheximide at a lower concentration (5 μg/ml) inhibits the ability of macrostomal cells to capture prey. Dichloroisoproterenol (50 μg/ml) and exogenous ATP, but not GTP, ITP, CTP, TTP, or UTP, stimulate rapid collapse of the cytopharyngeal pouch (Buhse *et al.,* 1978; Ryals and Yang, 1997). The percentage of a population with collapsed pouches is dependent on ATP concentration. The cation chelators EDTA and EGTA also induce cytopharyngeal pouch collapse and resorption (Sherman *et al.,* 1982).

The cytopharyngeal pouch can be manipulated in other ways that result in the synchronous sealing of the pouch in the absence of prey forming single, large, empty food vacuoles (Sherman *et al.,* 1981, 1982). Addition of 10 m*M* Ca^{2+}, 4 m*M* Ba^{2+}, or 4 m*M* Sr^{2+} will close the cytopharyngeal pouch to form a vacuole. However, 10 m*M* Na^{+} has no effect on pouch closure when used alone, but its presence reduces the requirement for calcium from 10 to 1 m*M*. Vacuolization of the pouch may also be achieved by the addition of the ionophore A-23187 (40 μM) or exposure to a 12-V electric current. After calcium-induced vacuole formation, a new cytopharyngeal pouch begins to form immediately, even in the presence of 10 m*M* Ca^{2+}. The macrostomal cell possessing a second cytopharyngeal pouch can be induced to form another empty vacuole by calcium only if the addition is preceded by washing the cells into calcium-free medium.

The size of the opening between the buccal cavity and the cytopharyngeal pouch and the ability to experimentally induce synchronous closure of the cytopharynx with sealing of the vacuole have permitted a detailed description of the structural changes that occur during this process (Buhse *et al.,* 1982; Smith-Somerville and Buhse, 1984). The cytostomal opening is made smaller by the lifting of the right and posterior ribbed walls and the movement of the left and anterior walls to the right. A diagonal furrow is formed when the walls meet. The fine filamentous reticulum, which borders the right, posterior and anterior margins of the cytostome–cytopharyngeal complex, is in an ideal position to bring the walls together by contraction (Fig. 5). Méténier (1984) reported that actin filaments are located parallel to the oral rib microtubles, below the membranelles, and in the specialized cytoplasm embedding the deep fiber in the oral apparatus of *T. paravorax* based on decoration with heavy meromyosin. Recently, an antibody to the calcium-binding cytoskeletal protein centrin has been shown to bind to the fine filamentous reticulum in both the microstomal and the macrostomal forms of *T. vorax* (McLaughlin *et al.,* 2000). All these results strongly indicate that a calcium-mediated contractile mechanism is responsible for cytopharynx closure and vacuole detachment.

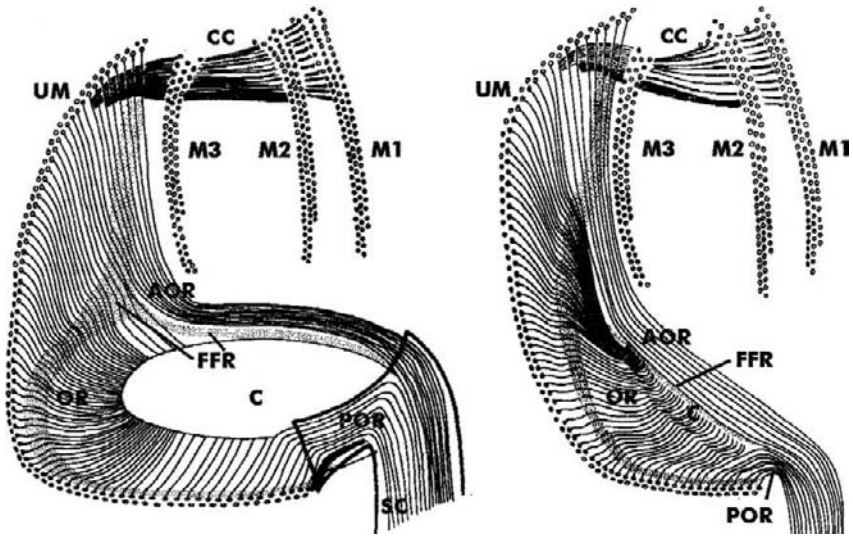


FIG. 5 Schematic diagram illustrating general arrangement of the anterior and posterior oral rib microtubules (AOR and POR) and the fine filamentous reticulum (FFR) when the cytostome–cytopharyngeal complex (C) is open (left) and closed (right). The AOR and POR are embedded in specialized cytoplasm (SC) in the region outlined. In the open cytostome–cytopharyngeal complex, the oral rib microtubules (OR) which line the right and posterior walls of the cytopharynx do not slope toward the center of the opening but are drawn this way to permit better visualization of the region. The cross-connective (CC) links membranelles 1–3 (M1–M3) with the undulating membrane (UM) (reproduced with permission from Smith, 1982b, Fig. 5, p. 375).

V. Phenotype Switching

Kidder and colleagues (1940) first reported the polymorphic nature of *T. vorax* strain D, the now extinct original isolate. They observed that large, carnivorous forms of *T. vorax* appeared when microstomal cells were placed in the presence of suitable prey; in this case, *Colpidium*. At that time, it was thought that living prey must be present to induce differentiation and that mechanical stimulation of the microstomal population was in some way involved. Later, Claff (1947) found that microstomal forms, when separated from the potential prey by a cellophane membrane, would undergo differentiation, indicating the existence of a diffusible substance originating with the prey population. This report was confirmed by Buhse (1966a,1967a), who named the diffusible agent "stomatin" because its initial observable effect on microstomal cells was manifested in rearrangement of the oral structure. Unfortunately, confusion was introduced into the literature when Stewart *et al.* (1993) gave the name stomatin to a protein from red blood cell membranes.

Buhse devised a method for producing large quantities of impure, but highly active, stomatin by concentrating the material contained in distilled water after incubating large, washed populations of *T. pyriformis,* one of several ciliates effective in inducing differentiation. Other ciliates producing active stomatin include *Colpidium* sp., *T. thermophila, T. membris,* and *Paramecium tetraurelia* (Butzel and Fischer, 1983; Grelland, 1988b; Kidder *et al.,* 1940; Smith-Somerville *et al.,* 2000).

A. What Is Stomatin?

Crude stomatin is pale yellow and contains numerous components, including sugars, amino acids, bases, and small amounts of chloroform/methanol extractable material composed of free fatty acids, phospholipids, and material that comigrates with tetrahymanol on thin layer plates (Butzel and Fischer, 1983; Ryals *et al.,* 1989; Smith-Somerville *et al.,* 2000). Buhse (1966a, 1967a) demonstrated that the activity was heat and cold stable and was not destroyed by various degradative enzymes, although RNAse at high concentration did reduce the activity. Tests using commercially obtained compounds proved ineffective in promoting macrostomal cell formation.

Butzel and Fischer (1983) reported that hypoxanthine was effective in generating large numbers of macrostomal cells, particularly when augmented by uridine. Hypoxanthine and either uridine or uracil have long been recognized as excretory products of nucleic acid catabolism in *Tetrahymena* (Butzel and Fischer, 1983; Leboy *et al.,* 1964) and are present in stomatin. Other laboratories were unable to duplicate their results (Grelland, 1988b; Smith-Somerville *et al.,* 2000), and it appeared that these two nucleic acid catabolites were not the sole active components of stomatin. Smith-Somerville *et al.* (2000) established the identity of the inducer as a complex of hypoxanthine, uracil, and ferrous iron using chromatographic and nuclear magnetic resonance methods. Ferrous iron is consistent with the observations that stomatin is most active at an acid pH (Buhse, 1966a). Pale yellow stomatin (consistent in color with solutions of ferrous iron) initiates high levels of transformation, whereas brown stomatin (characteristic of solutions of ferric iron) results in a significantly reduced response. Activity comparable to that of native stomatin was obtained with a synthetic chelate of hypoxanthine, uracil, and ferrous iron (Smith-Somerville *et al.,* 2000). Dissociation of the complex abolishes its signaling properties, but a synthetic preparation containing only hypoxanthine and ferrous iron has approximately half the activity of native stomatin. The stoichiometry of the complex remains undetermined because attempts to grow crystals for X-ray diffraction analysis have been unsuccessful. The coordination potential between hypoxanthine, uracil, and ferrous iron indicates that several combinations/arrangements could be formed. Whether this complex is assembled inside the cell prior to excretion or is formed after release of the individual components is also unknown. It is possible that this complex is involved in the transport and/or metabolism of iron.

B. Mechanism of Stomatin Action

Are there receptors for the inducer on the cell surface of polymorphic *Tetrahymena*? Although receptors have not been identified, a transmembrane signaling cascade does appear to be triggered in differentiation-competent cells (Ryals *et al.,* 1999). Pretreatment of *T. vorax* with several proteases resulted in a concentration-dependent reduction in the percentage of cells within a population that responded to stomatin (Table IV). This suggests that the presence of a surface-exposed protein is required for differentiation. Ryals and colleagues (1999) found that exposure to stomatin was followed by an increase in intracellular polyphosphoinositol concentration with a corresponding decrease in membrane phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 1,4,5-trisphosphate within 5 min. They also noted that the effects of inhibitors implicated involvement of several signaling cascade components in initiating transition to the macrostomal form.

Variations in the number of cell-surface receptors or in components of the signaling cascade could explain the differences in the D-type, M-type, and S-type sublines obtained from cultures of *T. vorax* V_2 (Shaw and Williams, 1963; Williams, 1961). These sublines differed in cell and nuclear size, meridian number, growth properties, serotype, and the ability to form macrostomal cells. Cultures of the original D-type subline contained quantities of both microstomal and macrostomal cells, whereas cultures of the original M- and S-type sublines consisted almost entirely of microstomal cells unless mixed with prey ciliates. These characteristics were clonally inherited. Lower temperatures and low pH favored higher percentages of differentiation in populations of D-type cells. A similar variation in the percentage of macrostomal cells with temperature and pH also occurs in stationary phase cultures of *T. patula* strain L-FF (Stone, 1963). It is possible that these cells were (are) more sensitive to small amounts of stomatin, or that substantial quantities of stomatin are constitutively released, resulting in self-induced differentiation.

C. Biochemical Alterations during Differentiation to the Macrostomal Cell Type

Progression from the microstomal to the macrostomal phenotype of *T. vorax* requires both transcription and translation (Table IV). Electrophoretic studies have generally revealed few discernable changes in unmodified protein profiles other than in polypeptides associated with isolated oral apparatuses from each cell type (Gulliksen *et al.,* 1984). Presumably, the altered protein composition of the macrostomal cell oral apparatus is necessary to accommodate the radical structural modifications that occur in that organelle. Recently, a putative differentiation-specific protein has been reported (Green *et al.,* 2000); this result awaits confirmation by other laboratories.

Marked changes are apparent, however, in the glycosyl phosphatidylinositol (GPI)-anchored proteins during differentiation (Yang and Ryals, 1994). GPI-anchored proteins with approximate molecular weights of 28, 50, and 82 kDa were observed in each cell type, but an additional protein of approximately 32 kDa appears during differentiation. Addition of D-mannosamine, which selectively blocked assembly of GPI-anchored proteins, inhibited the differentiation process. These results indicate that continuous GPI-anchored protein synthesis and assembly are essential to signal recognition- and/or differentiation-related processes occurring downstream of the initial interaction of the signal with the cell surface. Several studies have demonstrated a requirement for ongoing synthesis of total protein during the formation of macrostomal cell types (Buhse, 1967b; Buhse and Hamburger, 1974). This study of GPI-anchored proteins, however, demonstrated the importance of a small subset of cellular polypeptides.

In addition to the appearance of a new GPI-anchored protein, differential radiolabeling indicates that targeted retailoring of the fatty acids associated with the GPI anchors of the 28- and 32-kDa proteins occurs during the development of the macrostomal form (Yang and Ryals, 1994). The significance of this retailoring event is not understood, although subtle changes in the fatty acid composition of the anchor moieties might allow for a more secure attachment of the anchored proteins to the lipid bilayer. Microstomal cells exhibit a different degree of membrane fluidity compared to macrostomal cells (Ryals and Smith-Somerville, 1986). These cells also possess an array of other lipid-modified proteins, including myristoylated, palmitoylated, and prenylated proteins (Ryals, 1996; Yang and Ryals, 1994); however, no significant changes in these molecules as a result of differentiation have been demonstrated.

Western blots probed with anti-phospho-amino acid antibodies have been employed with only limited success to investigate whether changes in protein phosphorylation occur during differentiation (P. Ryals, unpublished observations). These preliminary observations suggest that several proteins are dephosphorylated soon after the addition of stomatin to cells. Changes in protein phosphorylation during the first 5 min following stomatin addition were not examined as part of this study; therefore, early activation of kinase in response to the interaction of stomatin with its putative receptor has yet to be investigated. Relatively weak increases in the level of phosphorylation were observed in two phosphoserine-containing polypeptides, a phosphotyrosine-containing polypeptide, and a phosphothreonine-containing polypeptide having estimated molecular weights of 70, 27, 21, and 36 kDa, respectively. Interestingly, these apparent changes in protein phosphorylation coincide temporally with the formation of the cytopharyngeal pouch 6 h following stomatin addition.

The initial transition events include ubiquitin-mediated protein degradation. MG 115, a potent reversible inhibitor of the 20S and 26S proteasomes (Rock *et al.,* 1994), blocks differentiation in *T. vorax* (H. Smith-Somerville, unpublished observations). This peptide aldehyde also inhibits lysosomal cysteine proteases,

but phenylmethylsulphonyl fluoride, a cysteine protease inhibitor, has no effect. Thus, the inhibitory effect appears to result from a block in proteasome degradation rather than inhibition of lysosomal degradation. Pre-treatment of cells with MG 115 followed by the addition of stomatin with the inhibitor was most effective.

The predominant phospholipids found in both microstomal and macrostomal cells of *T. vorax* are phosphatidylethanolamine and aminoethylphosphonolipid, with small amounts of phosphatidylcholine, diphosphatidylglycerol (cardiolipin), and sphingolipid (Ryals *et al.,* 1989). The percentage distribution of fatty acid or acetate radioactivity into each class of phospholipid is similar to the distributions reported for nondifferentiating species of *Tetrahymena,* and only small variations in radiolabeled fatty acid distribution were observed between microstomal and macrostomal phenotypes. *Tetrahymena vorax* also contains small amounts of phosphatidyl-*myo*-inositol, phosphatidyl-*myo*-inositol bisphosphate, and phosphatidyl-*myo*-inositol trisphosphate (Ryals *et al.,* 1999). Interestingly, when *T. vorax* is presented with [^{3}H]*scyllo*-inositol, the cells utilize it in the biosynthesis of phosphatidyl-*scyllo*-inositol and *scyllo*-inositol-containing PIP and PIP_2 (Ryals and Kersting, 1999). The physiological significance of these lipids is not known, but they or their hydrolysis products may possess unique second messenger properties.

Analysis of total phospholipid-bound fatty acids of microstomal and macrostomal cell types revealed a high proportion of unsaturated C_{18} fatty acids, typical of other *Tetrahymena* species (Ryals *et al.,* 1989). Macrostomal cells have significantly increased amounts of *cis*-$18{:}3^{(\Delta 6,9,12)}$ (γ-linolenic acid) compared to their microstomal counterparts. The whole cell phospholipid unsaturated to saturated fatty acid ratio increased from 4.6 in microstomal cells to 5.5 in macrostomal cells, but the unsaturated to saturated ratio (1.4) for *T. vorax* triacylglycerol-bound fatty acids was the same in both phenotypes. All *Tetrahymena* lack sterols but contain small amounts of the pentacyclic triterpenoid, tetrahymanol. Glyceryl ether lipids are also characteristically found in *Tetrahymena* species.

Fatty acids from whole cells of *T. paravorax* RP have been analyzed under growth conditions similar to those used for *T. vorax* (Erwin and Bloch, 1963). *Tetrahymena paravorax* lipids were reported to contain a relatively large amount (15%) of C_{17} (isoheptadecanoic + isoheptadecenoic acid). Microstomal cells of *T. paravorax* also contain somewhat less $C_{18:3}$ than *T. vorax*. Lipids in the macrostomal form of *T. paravorax* were not characterized.

D. Divide or Differentiate?

Early studies on the polymorphic properties of *T. vorax* indicated that sublines differ significantly in the cell type present under different growth conditions. In *T. vorax* V_2S, the microstomal form is the predominant cell type under optimum culture conditions in Loefer's medium (Loefer *et al.,* 1958), iron-enriched

proteose peptone medium (Nelsen *et al.,* 1981; Thompson, 1967), or defined medium (Holz *et al.,* 1962; Szablewski *et al.,* 1991). Other sublines that either regularly have substantial numbers of both macrostomes and microstomes present in axenic stationary-phase cultures or are low-transforming sublines have arisen spontaneously (Grelland, 1988a,b; Ryals and Smith-Somerville, 1985, 1986; Shaw and Williams, 1963; Williams, 1961). Stone (1963) reported that the predominant phenotype of *T. patula* L-FF during logarithmic growth in Loefer's medium is also the microstomal cell, but during stationary phase populations can consist entirely of macrostomal cells depending on the temperature and pH of the medium. Logarithmic- or stationary-phase cultures in iron-enriched proteose peptone medium are essentially all macrostomal cells (P. Ryals, unpublished observations). This observation reinforces the idea that the presence and oxidation state of iron are influential in determining the phenotype of the cell. In 1973, Frankel and Williams proposed that a nutritional change destabilizes the metastatic oral structure and triggers oral morphogenesis leading to either division or oral replacement that occurs in phenotype switching. With the identification of a specific induction factor present in stomatin and advances in understanding cell cycle regulation, this idea has evolved, and we now suggest an interpretation that deemphasizes the broad concept of nutritional control and concentrates instead on the relationship between the cell cycle and differentiation.

The presence of a competence point for differentiation during the cell cycle was suggested by Buhse and Rasmussen (1974) for *T. vorax* and by Méténier and collaborators (Méténier, 1976, 1978; Méténier and Grolière, 1979) for *T. paravorax*. The highest percentages of macrostomal cells develop in populations with the greatest degree of synchrony, and the time required to reach maximum differentiation is equivalent to the time of the division cycle. The original proposal arose from the demonstration that high percentages of macrostomal cells could be obtained by transferring *T. vorax* to inorganic medium (Hamburger and Zeuthen, 1957) after a series of heat shocks, a method used to synchronize cell division in *Tetrahymena* (Buhse and Rasmussen, 1974; Buhse *et al.,* 1974; Buhse and Zeuthen, 1974; Scherbaum and Zeuthen, 1954; Williams, 1964). Both Buhse (1966a) and Grelland (1988b) reported that the potential for differentiation was dependent on culture age. Maximum percentages of transformed cells occur when initiated in populations of microstomal cells in late logarithmic or early stationary phases of growth when the cells exhibit the greatest degree of division synchrony (Buhse, 1966a; Grelland, 1986, 1988b). Attempts to enhance phenotype switching in low-differentiating sublines led to the identification of an array of compounds that substantially boosted differentiation in heat-shocked populations (Ryals and Smith-Somerville, 1985, 1986). These included α-tocopheryl succinate (vitamin E), phylloquinone (vitamin K), retinol palmitate and coenzyme Q_9, several alcohols, and the higher oxidation states of iron and copper. All of these "enhancers" appeared to act by delaying cell division following heat shock, probably generating greater synchrony in a population and thus a greater number of differentiation-competent cells.

Not only can heat shock serve as an alternative to stomatin for initiation of phenotype switching but also elevated temperature treatment affects the normal progression of the differentiation process in stomatin-induced cells. Differentiation is delayed in cells heat shocked shortly after exposure to stomatin relative to non-heat-shocked controls (Holsen and Buhse, 1969). The delay in differentiation increases with increasing population age, suggesting that the effect is dependent on the growth phase of the population. Populations of stomatin-treated cells divide when heat shocked at the midpoint of phenotype switching. Whereas heat shocks administered after the midpoint have no effect on either the degree or the timing of differentiation. Among the established effects of heat shock on cells are inhibition of transcription, translation, and RNA processing; altered protein degradation; inhibition or activation of kinases and phosphatases; and effects on membrane structure and function, ion concentrations, energy metabolism, and stability of the cytoskeleton (Kühl and Rensing, 2000). There is an apparent requirement for specific environmental and intracellular conditions at multiple checkpoints in the cell cycle or the differentiation process. Some heat labile event critical to macrostomal cell formation occurs in this window of time, and if prevented the cells revert to the default division pathway.

E. How Does It Work in the Pond?

Upon first consideration, it is difficult to understand how polymorphic *Tetrahymena,* in the relative vastness of a natural aqueous ecosystem, accomplish the feat of morphing into the carnivorous macrostomal form. How could conditions favorable to differentiation accumulate to trigger this response? The following scenario could occur in relatively still water such as a pond, small inlet, or standing pool. The microstomal cell type and all its potential prey compete for the same particulate food source (bacteria) utilizing an oral apparatus designed to exclusively harvest and concentrate bacteria-sized particles. The potential predator is eventually outnumbered and outcompeted by its faster dividing protist neighbors. This marks the onset of conditions conducive to differentiation. Particulate food becomes scarce as larger numbers of bacteria are consumed and the onset of starvation conditions (or near-starvation conditions) begins. Relative pH begins to decline, as supported by *in vitro* studies that show that water containing *T. vorax* becomes progressively more acidic with time (P. Ryals and H. Smith-Somerville, unpublished observation). As the number of potential prey ciliates increases, the concentration of the inducer in the environment increases. Dilution and the instability of the stomatin complex under conditions of ambient temperature and near neutral pH would limit the persistence of the differentiation signal; however, sensitive microstomal cells would respond if in close proximity to the prey. These cells develop into carnivores, utilizing an altered oral apparatus that is only capable of ingesting prey and incapable of sequestering bacteria. They feed on the prey protists until the numbers

of prey diminish, the concentration of the triggering complex declines, and the macrostomal cells divide forming microstomal cells once again. The competition for bacteria begins anew as the bacteria are freed from feeding pressure and their numbers again increase.

VI. Phenotype Switching in Other Organisms

Phenotype switching of an organism in response to a prey/predator interaction is not limited to polymorphic species of *Tetrahymena* but, rather, is widespread in nature. Another ciliate, *Blepharisma americanum,* develops a larger oral apparatus in response to α-tocopherol (Lennartz and Bovee, 1980). Interestingly, this vitamin enhances differentiation in certain populations of *T. vorax* (Ryals and Smith-Somerville, 1985). In most cases, however, the alterations appear as defensive changes in the prey to prevent ingestion by the predator. Well-studied examples among the ciliates are species of the genus *Euplotes* that alter their cellular architecture in response to morphogens released by predators, including the ciliate *Lembadion bullinum* (Kusch and Heckmann, 1992), the rhizopod *Amoeba proteus* (Kusch, 1993a), and the turbellarian *Stenostomum sphagnetorum* (Kusch, 1993b). Other documented changes in morphology include an antipredator morphology adopted by *Daphnia pulex* in response to a kariomone released by the midge *Chaoborus americanus* (Parejko and Dodson, 1990; Tollrian and von Elert, 1994) and spine development in the rotifer *Brachionus calcifours* induced by a factor released by the rotifer *Asplanchnia brightwelli* (Gilbert, 1966).

VII. Concluding Remarks

Phenotype switching in polymorphic *Tetrahymena* shares many fundamental similarities with differentiation in higher organisms. As stated by Trager (1963), "the essential aspect of differentiation is change—change in the chemistry and morphology of a cell making it distinctly different from its previous state." This change is dependent on environmental signals, including nutrient availability, and temperature and change in pH brought about by cellular growth. Cell–cell signaling occurs wherein prey ciliates (signaling cells) release a low-molecular-weight metallocomplex which is recognized by the potential predator (target cells) as a signal to differentiate into the carnivorous form. Furthermore, the signal is recognized by binding to a putative receptor on the cell surface, activating a signal pathway leading to polyphosphoinositide hydrolysis and subsequent second messenger production. Transformation results in a change in form leading to a change in function, specifically resorption and replacement of the oral apparatus and formation

of a cytopharyngeal pouch designed to capture and digest potential prey. These changes are scripted at the molecular level by selected gene activation as evidenced by the requirement for transcription and translation. Macrostomal cell formation is dependent on completion of a critical event that renders the cell competent to differentiate until approximately midway through the differentiation cycle. If this event is blocked, the cells default to the division pathway. These shared characteristics make studies of phenotype switching in the polymorphic species applicable to elucidation of fundamental cellular developmental processes. Important questions regarding the biogenesis of the signaling molecule, its bioactive structure, and the characterization of the complete signaling pathway remain to be answered.

Acknowledgments

We are grateful to Michael C. Kersting, Suzanne McCutcheon, and Neil McLaughlin for their comments and careful reading of the manuscript. We particularly thank our colleagues and students who contributed to the original work. The research has been supported by grants from the National Science Foundation, the Edna McConnell Clark Foundation, and the Mississippi Agricultural and Forestry Experiment Station (current Project No. MIS-701120 to P.E.R.). The authors dedicate this review to Dr. Norman E. Williams on the occasion of his retirement.

References

Allen, R. D. (1967). Fine structure, reconstruction and possible functions of components of the cortex of *Tetrahymena pyriformis*. *J. Protozool.* **14,** 553–565.

Allen, R. D., and Fok, A. K. (2000). Membrane trafficking and processing in *Paramecium*. *Int. Rev. Cytol.* **198,** 277–318.

Anderson, R. G., Kamen, B. A., Rothberg, K. G., and Lacey, S. W. (1992). Potocytosis: Sequestration and transport of small molecules by caveolae. *Science* **255,** 410–411.

Aufderheide, K. (1979). Mitochondrial associations with specific microtubular components of the cortex of *Tetrahymena thermophila*. I. Cortical patterning of mitochondria. *J. Cell Sci.* **39,** 299–312.

Brunk, C. F., Kahn, R. W., and Sadler, L. A. (1990). Phylogenetic relationships among *Tetrahymena* species determined using the polymerase chain reaction. *J. Mol. Evol.* **30,** 290–297.

Buhse, H. E., Jr. (1966a). An analysis of macrostome production in *Tetrahymena vorax* strain V_2S-type. *J. Protozool.* **13,** 429–435.

Buhse, H. E., Jr. (1966b). Oral morphogenesis during transformation from microstome to macrostome and macrostome to microstome in *Tetrahymena vorax* strain V_2 type S. *Trans. Am. Microsc. Soc.* **85,** 305–313.

Buhse, H. E., Jr. (1967a). Microstome–macrostome transformation in *Tetrahymena vorax* strain V_2 type S induced by a transforming principle, stomatin. *J. Protozool.* **14,** 608–613.

Buhse, H. E., Jr. (1967b). Studies on the initiation and execution of microstome → macrostome transformation in *Tetrahymena vorax*. *Trans. Am. Microsc. Soc.* **86,** 65–66.

Buhse, H. E., Jr., and Cameron, I. L. (1968). Temporal pattern of macronuclear events during microstome–macrostome cell transformation of *Tetrahymena vorax* strain V_2S. *J. Exp. Zool.* **169,** 229–236.

Buhse, H. E., Jr., and Hamburger, K. (1974). Induced macrostome formation in *Tetrahymena vorax* strain V_2: Patterns of respiration. *C. R. Trav. Lab. Carlsberg* **40,** 77–89.

Buhse, H. E., Jr., and Nicolette, J. A. (1969). A possible role of proteins in the regulation of RNA synthesis in *Tetrahymena vorax*. *In* "Progress in Protozoology. Proceedings of the 3rd International Congress on Protozoology" (K. Strelkov, M. Sukhanova, and I. B. Raikov, Eds.), pp.136–137. Publishing House Nauka, Leningrad.

Buhse, H. E., Jr., and Rasmussen, L. (1974). A method for synchronization of cell division and macrostome formation in the ciliate *Tetrahymena vorax*. *C. R. Trav. Lab. Carlsberg* **40,** 59–67.

Buhse, H. E., Jr., and Williams, N. E. (1982). A comparison of cortical proteins in *Tetrahymena vorax* microstomes and macrostomes. *J. Protozool.* **29,** 222–226.

Buhse, H. E., Jr., and Zeuthen, E. (1974). Oral morphogenesis in *Tetrahymena* cells synchronized with one heat shock per generation. *C. R. Trav. Lab. Carlsberg* **39,** 493–505.

Buhse, H. E., Jr., Corliss, J. O., and Holsen, R. C., Jr. (1970). *Tetrahymena vorax:* Analysis of stomatogenesis by scanning electron and light microscopy. *Trans. Am. Microsc. Soc.* **89,** 328–336.

Buhse, H. E., Jr., Hamburger, K., and Lykkesfeldt, A. (1974). Respiration and macromolecular synthesis during cell division and macrostome formation in heat synchronized *Tetrahymena vorax,* strain V_2. *C. R. Trav. Lab. Carlsberg* **40,** 69–75.

Buhse, H. E., Jr., Smith, H. E., Sonkin, D. J., and Fleming, J. J. (1978). Some factors which affect the maintenance and shape of the cytopharyngeal pouch in carnivorous forms of *Tetrahymena vorax*. *Trans. Am. Microsc. Soc.* **97,** 350–362.

Buhse, H. E., Jr., Smith, H. E., and Sherman, G. B. (1982). Scanning electron microscopic examination of Ca^{2+}-induced food vacuole formation in the carnivorous macrostomal form of *Tetrahymena vorax,* strain V_2S. *Scanning Electron Microsc.* **4,** 1661–1666.

Buhse, H. E., Jr., Nabazas, S., Buhrfeind, C., and Ryals, P. E. (1991). The effect of the lipogenic inhibitor cerulenin, on macrostomal cell formation in *Tetrahymena vorax*. *J. Protozool.* **38,** 124–129.

Butzel, H. M., and Fischer, J. (1983). The effects of purine and pyrimidines upon transformation in *Tetrahymena vorax*, strain *V_2S*. *J. Protozool.* **30,** 247–250.

Cassidy-Hanley, D., Smith, H. R., and Bruns, P. J. (1995). A simple, efficient technique for freezing *Tetrahymena thermophila*. *J. Eukaryot. Microbiol.* **42,** 510–515.

Claff, C. L. (1947). Induced morphological changes in *Tetrahymena vorax*. *Biol. Bull.* **93,** 216–217.

Collins, T., Baker, R. L., Wilhelm, J. M., and Olmsted, J. B. (1980). A cortical scaffold in the ciliate *Tetrahymena*. *J. Ultrastruct. Res.* **70,** 92–103.

Conner, R. L., and Koroly, M. J. (1973). Chemistry and metabolism of nucleic acids in *Tetrahymena*. *In* "Biology of *Tetrahymena*" (A. M. Elliott, Ed), pp. 123–164. Dowden, Hutchinson & Ross, Stroudsburg, PA.

Corliss, J. O. (1953). Comparative studies on holotrichous ciliates in the *Colpidium–Glaucoma–Leucophrys–Tetrahymena* group. II. Morphology, life cycles and systematic status of strains in pure culture. *Parasitology* **43,** 49–87.

Corliss, J. O. (1957). *Tetrahymena paravorax* n. sp., the first caudal-ciliated member of the genus referable to the *vorax–patula* complex. *J. Protozool.* **4**(Suppl.), 13.

Corliss, J. O. (1973). History, taxonomy, ecology, and evolution of species of *Tetrahymena*. *In* "Biology of *Tetrahymena*" (A. M. Elliott, Ed.), pp. 1–55. Dowden, Hutchinson & Ross, Stroudsburg, PA.

Dragesco, J., and Njiné, T. (1971). Compléments à la connaissance des ciliés libres du Cameroun. *Ann. Fac. Sci. Cameroun* **7–8,** 97–140.

Elliott, A. M., and Hayes, R. E. (1954). A conjugating strain of *Tetrahymena vorax*. *Biol. Bull.* **107,** 309.

Elliott, A. M., and Kennedy, J. R. (1973). Morphology of *Tetrahymena*. In "Biology of *Tetrahymena*" (A. M. Elliott, Ed.), pp. 57–87. Dowden, Hutchinson & Ross, Stroudsburg, PA.

Erwin, J. A., and Bloch, K. (1963). Lipid metabolism of ciliated protozoa. *J. Biol. Chem.* **238,** 1618–1624.

Frankel, J. (1983). What are the developmental underpinnings of evolutionary changes in protozoan morphology. *In* "Development and Evolution" (B. C. Goodwin, N. Holder, and C. G. Wylie, Eds.), pp. 279–314. Cambridge Univ. Press, London.

Frankel, J. (1989). "Pattern Formation. Ciliate Studies and Models," Oxford Univ. Press, New York.

Frankel, J., and Williams, N. E. (1973). Cortical development in *Tetrahymena*. *In* "Biology of *Tetrahymena*" (A. M. Elliott, Ed.), pp. 375–409. Dowden, Hutchinson & Ross, Stroudsburg, PA.

Furgason, W. H. (1940). The significant cytostomal pattern of the "*Glaucoma–Colpidium* group," and a proposed new genus and species, *Tetrahymena geleii*. *Arch. Protistenkd.* **94,** 224–266.

Gabe, P. R., and Williams, N. E. (1982). The formation and fate of *Tetrahymena patula* cryptostomes. *J. Protozool.* **29,** 2–7.

Gilbert, J. J. (1966). Rotifer ecology and embryological induction. *Science* **151,** 1234–1237.

Green, M. M., LeBoeuf, R. D., and Churchill, P. F. (2000). Biological and molecular characterization of cellular differentiation in *Tetrahymena vorax*: A potential biocontrol protozoan. *J. Basic Microbiol.* **40,** 351–361.

Grelland, E. M. (1986). Growth control by G2-arrest in batch cultures of *Tetrahymena vorax*. *Protistologica* **22,** 245–256.

Grelland, E. M. (1988a). Different growth phenotypes of *Tetrahymena vorax* V_2 microstomes. I: Changed control of cell cycle passage. *Eur. J. Protistol*. **23,** 309–316.

Grelland, E. M. (1988b). Different growth phenotypes of *Tetrahymena vorax* V_2 microstomes. II: Kinetics of macrostome differentiation. *Eur. J. Protistol*. **24,** 52–59.

Gulliksen, O. M., Løvlie, A., and Kvammen, L. (1984). Different polypeptides in two homologous cellular structures: The microstomal and macrostomal oral apparatus of *Tetrahymena vorax*. *Dev. Biol.* **103,** 511–516.

Hamburger, K., and Zeuthen, E. (1957). Synchronous divisions in *Tetrahymena pyriformis* as studied in an inorganic medium. The effect of 2,4-dinitrophenol. *Exp. Cell Res.* **13,** 443–453.

Holsen, R. C., Jr., and Buhse, H. E., Jr. (1969). The effect of heat shocks on the morphogenetic events associated with macrostome formation in *Tetrahymena vorax*. *In* "Progress in Protozoology. Proceedings of the 3rd International Congress on Protozoology" (K. Strelkov, M. Sukhanova, and I. B. Raikov, Eds.), pp. 97–98. Publishing House Nauka, Leningrad.

Holz, G. G., Jr. (1964). Nutrition and metabolism of ciliates. *In* "Biochemistry and Physiology of Protozoa" (S. H. Hutner, Ed.), Vol. **3,** pp. 199–242. Academic Press, New York.

Holz, G. G., Jr. (1973). The nutrition of *Tetrahymena:* Essential nutrients, feeding and digestion. *In* "Biology of *Tetrahymena*" (A. M. Elliott, Ed.), pp. 89–98. Dowden, Hutchinson & Ross, Stroudsburg, PA.

Holz, G. G., Jr., Erwin, J. A., and Wagner, B. (1961). The sterol requirement of *Tetrahymena paravorax* RP. *J. Protozool.* **8,** 297–300.

Holz, G. G., Jr., Erwin, J. A., Rosenbaum, N., and Aaronson, S. (1962). Triparanol inhibition of *Tetrahymena* and its prevention by lipids. *Arch. Biochem. Biophys.* **98,** 312–322.

Kidder, G. W., and Dewey, V. C. (1951). The biochemistry of ciliates in pure culture. *In* "Biochemistry and Physiology of Protozoa" (A. Lwoff, Ed.), Vol. **1,** pp. 323–400. Academic Press, New York.

Kidder, G. W., Lilly, D. M., and Claff, C. L. (1940). Growth studies on ciliates. IV. The influence of food on the structure and growth of *Glaucoma vorax* sp. nov. *Biol. Bull.* **78,** 9–23.

Kühl, N. M., and Rensing, L. (2000). Heat shock effects on cell cycle progression. *Cell. Mol. Life Sci.* **57,** 450–463.

Kurz, S., and Tiedtke, A. (1993). The Golgi apparatus of *Tetrahymena thermophila. J. Eukaryot. Microbiol.* **40,** 10–13.

Kusch, J. (1993a). Behavorial and morphological changes in ciliates induced by the predator *Amoeba proteus*. *Oecologia* **96,** 354–359.

Kusch, J. (1993b). Predator-induced morphological changes in *Euplotes* (Ciliata): Isolation of the inducing substance released from *Stenostomum sphagnetorum* (Turbellaria). *J. Exp. Zool.* **265,** 613–618.

Kusch, J., and Heckmann, K. (1992). Isolation of the *Lembadion*-factor, a morphogenetically active signal, that induces *Euplotes* cells to change from their ovoid form into a larger lateral winged morph. *Dev. Genet.* **13,** 241–246.

Lascelles, J. (1957). Synthesis of porphyrins by cell suspensions of *Tetrahymena vorax:* Effect of members of the vitamin B group. *Biochem. J.* **66,** 65–72.

Leboy, P. S., Cline, S. G., and Conner, R. L. (1964). Phosphate, purines and pyrimidines as excretory products of *Tetrahymena*. *J. Protozool.* **11,** 217–222.

Lennartz, D. C., and Bovee, E. C. (1980). Induction of macrostome formation in *Blepharisma americanum* (Suzuki, 1954) by alpha-tocopheryl succinate. *Trans. Am. Microsc. Soc.* **99,** 310–317.

Loefer, J. B., Owen, R. D., and Christensen, E. (1958). Serological types among thirty-one strains of the ciliated protozoan *Tetrahymena pyriformis*. *J. Protozool.* **5,** 209–217.

Luksas, A. J., and Erwin, J. A. (1983). The lipid requirement of the ciliated protozoon *Tetrahymena patula*. *J. Protozool.* **30,** 8–13.

McLaughlin, N. B., McCutcheon, S. M., Buhse, H. E., Jr., and Smith-Somerville, H. E. (2001). Identification of centrin in *Tetrahymena vorax*. *J. Eukaryot. Microbiol.* **48,** 8A.

Méténier, G. (1976). Influence de la densité cellulaire, de l'âge des cultures et de la "stomatine" sur le rendement de la transformation microstome–macrostome chez *Tetrahymena paravorax*. *Protistologica* **12,** 389–398.

Méténier, G. (1978). Preuves cinétiques de l'existence d'une période de compétence pour la transformation microstome–macrostome dans le cycle cellulaire de *Tetrahymena paravorax*. *J. Protozool.* **25,** 326–334.

Méténier, G. (1984). Actin in *Tetrahymena paravorax:* Ultrastructural localization of HMM-binding filaments in glycerinated cells. *J. Protozool.* **31,** 205–215.

Méténier, G., and Grolière, C.-A. (1979). Précisions temporelles sur les processus de la stomatogenèse de *Tetrahymena paravorax* analysés en microscopie photonique. *J. Protozool.* **26,** 75–82.

Miller, O. L., Jr., and Stone, G. E. (1963). Fine structure of the oral area of *Tetrahymena patula*. *J. Protozool.* **10,** 280–288.

Mislan, T. W., and Smith-Somerville, H. E. (1986). Food vacuole morphology and membrane retrieval in the microstomal form of *Tetrahymena vorax*. *J. Protozool.* **33,** 172–179.

Nanney, D. L., Meyer, E. B., Simon, E. M., and Preparata, R.-M. (1989). Comparison of ribosomal and isozymic phylogenies of tetrahymenine ciliates. *J. Protozool.* **36,** 1–8.

Nanney, D. L., Park, C., Preparata, R., and Simon, E. M. (1998). Comparison of sequence differences in a variable 23S rRNA domain among sets of cryptic species of ciliated protozoa. *J. Eukaryot. Microbiol.* **45,** 91–100.

Nelsen, E. M., Frankel, J., and Martel, E. (1981). Development of the ciliature of *Tetrahymena thermophila*. I. Temporal coordination with oral development. *Dev. Biol.* **88,** 27–38.

Nicolette, J. A., Buhse, H. E., Jr., and Robin, M. S. (1971). The effect of 2-mercapto-1-(beta-4-pyridethyl) benzimidazole (MPB) on cell differentiation and RNA synthesis in the protozoon *Tetrahymena vorax*. *J. Protozool.* **18,** 87–90.

Nilsson, J. R. (1979). Phagotrophy in *Tetrahymena*. *In* "Biochemistry and Physiology of Protozoa" (M. Levandowsky and S. H. Hutner, Eds.), 2nd ed., Vol. **2,** pp. 339–379. Academic Press, New York.

Nilsson, J. R., and van Deurs, B. (1983). Coated pits and pinocytosis in *Tetrahymena*. *J. Cell Sci.* **63,** 209–222.

Njiné, T. (1972). La transformation microstome–macrostome et macrostome–microstome chez *Tetrahymena paravorax* Corliss 1957. *Ann. Fac. Sci. Cameroun* **10,** 69–84.

Parejko, K., and Dodson, S. (1990). Progress towards characterization of a predator/prey Kairomone: *Daphnia pulex* and *Chaoborus americanus*. *Hydrobiologia* **198,** 51–59.

Pitelka, D. R. (1961). Fine structure of the silverline and fibrillar system of three tetrahymenid ciliates. *J. Protozool.* **8,** 75–89.

Preparata, R. M., Meyer, E. B., Preparata, F. P., Simon, E. M., Vossbrinck, C. R., and Nanney, D. L. (1989). Ciliate evolution: The ribosomal phylogenies of the tetrahymenine ciliates. *J. Mol. Evol.* **28,** 427–441.

Rasmussen, L., Buhse, H. E., Jr., and Groh, K. (1975). Efficiency of filter feeding in two species of *Tetrahymena*. *J. Protozool.* **22,** 110–111.

Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class 1 molecules. *Cell* **78,** 761–771.

Ryals, P. E. (1996). Protein prenylation in *Tetrahymena*. *Arch. Protistenkd.* **147,** 199–204.

Ryals, P. E., and Kersting, M. C. (1999). Sodium-dependent uptake of [^{3}H]*scyllo*-inositol by *Tetrahymena:* Incorporation into phosphatidylinositol, phosphatidylinositol-linked glycans, and polyphosphoinositols. *Arch. Biochem. Biophys.* **366,** 261–266.

Ryals, P. E., and Smith-Somerville, H. E. (1985). Enhancement of macrostome formation in a low-transforming subline of *Tetrahymena vorax* by alpha-tocopheryl succinate and related compounds. *Trans. Am. Microsc. Soc.* **104,** 341–349.

Ryals, P. E., and Smith-Somerville, H. E. (1986). Studies of macrostome formation of low-transforming *Tetrahymena vorax*. Transformation enhancers, generation time and membrane fluidity. *J. Protozool.* **33,** 382–387.

Ryals, P. E., and Yang, X. (1997). Effects of dichloroisoproterenol on macromolecular synthesis and differentiation in *Tetrahymena vorax*. *J. Eukaryot. Microbiol.* **44,** 43–45.

Ryals, P. E., Buhse, H. E., Jr., and Modzejewski, J. (1989). Lipid modification during cytodifferentiation of *Tetrahymena vorax*. Whole cell phospholipids and triacylglycerols of microstomal and macrostomal phenotypes. *Biochim. Biophys. Acta* **991,** 438–444.

Ryals, P. E., Bae, S., and Patterson, C. E. (1999). Evidence for early signaling events in stomatin-induced differentiation of *Tetrahymena vorax*. *J. Eukaryot. Microbiol.* **46,** 77–83.

Sadler, L. A., and Brunk, C. F. (1992). Phylogenetic relationships and unusual diversity in histone H4 proteins within the *Tetrahymena pyriformis* complex. *Mol. Biol. Evol.* **9,** 70–84.

Satir, B. H., and Wissig, S. L. (1982). Alveolar sacs of *Tetrahymena:* Ultrastructural characteristics and similarities to subsurface cisterns of muscle and nerve. *J. Cell Sci.* **55,** 13–33.

Scherbaum, O., and Zeuthen, E. (1954). Induction of synchronous cell division in mass cultures of *Tetrahymena piriformis*. *Exp. Cell Res.* **6,** 221–227.

Schousboe, P., Christensen, S. T., Ghiladi, M., and Rasmussen, L. (1992). Porphyrin rings and phospholipids: Stimulators of cloning efficiency in certain species of *Tetrahymena*. *J. Protozool.* **39,** 343–345.

Shaw, R. F., and Williams, N. E. (1963). Physiological properties of *Tetrahymena vorax*. *J. Protozool.* **10,** 486–491.

Sherman, G. B., Buhse, H. E., Jr., and Smith, H. E. (1981). A method for synchronous induction of food vacuoles in the macrostome form of *Tetrahymena vorax*. *Trans. Am. Microsc. Soc.* **100,** 366–372.

Sherman, G. B., Buhse, H. E., Jr., and Smith, H. E. (1982). Physiological studies on the cytopharyngeal pouch, a prey receptacle in the carnivorous macrostomal form of *Tetrahymena vorax*. *J. Protozool.* **29,** 360–365.

Simon, E. M., Meyer, E. B., and Preparata, R. M. (1985). New wild *Tetrahymena* from Southeast Asia, China, and North America, including *T. malaccensis, T. asiatica, T. nanneyi, T. caudata,* and *T. silvana* n. spp. *J. Protozool.* **32,** 183–189.

Smith, H. E. (1982a). Oral apparatus structure in the microstomal form of *Tetrahymena vorax*. *Trans. Am. Microsc. Soc.* **101,** 36–58.

Smith, H. E. (1982b). Oral apparatus structure in the carnivorous macrostomal form of *Tetrahymena vorax*. *J. Protozool.* **29,** 616–627.

Smith, H. E., and Buhse, H. E., Jr. (1983). On the origin of the deep fiber in *Tetrahymena*. *Trans. Am. Microsc. Soc.* **102,** 264–271.

Smith-Somerville, H. E. (1989). Correlation of digestive stage with changes in amphiphilic phagosomal proteins of *Tetrahymena vorax*. *Eur. J. Cell Biol.* **49,** 48–54.

Smith-Somerville, H. E., and Buhse, H. E., Jr. (1984). Changes in oral apparatus structure accompanying vacuolar formation in the macrostomal form of *Tetrahymena vorax.* A model for the formation of food vacuoles in *Tetrahymena*. *J. Protozool.* **31,** 373–380.

Smith-Somerville, H. E., Verchot, M. E., and Ryals, P. E. (1986). Food vacuoles during heat-shock-induced transformation from the microstomal to the macrostomal cell type of *Tetrahymena vorax*. *J. Protozool.* **33,** 261–266.

Smith-Somerville, H. E., Huryn, V. B., Walker, C., and Winters, A. L. (1991). Survival of *Legionella pneumophila* in the cold-water ciliate *Tetrahymena vorax*. *Appl. Environ. Microbiol.* **57,** 2742–2749.

Smith-Somerville, H. E., Hardman, J. K., Timkovich, R., Ray, W. J., Rose, K. E., Ryals, P. E., Gibbons, S. H., and Buhse, H. E., Jr. (2000). A complex of iron and nucleic acid catabolites is a signal that triggers differentiation in a freshwater protozoan. *Proc. Natl. Acad. Sci. USA* **97,** 7325–7330.

Stewart, G. W., Argent, A. C., and Dash, B. C. (1993). Stomatin: A putative cation transport regulator in the red cell membrane. *Biochim. Biophys. Acta* **1225,** 15–25.

Stone, G. E. (1963). Polymorphic properties of *Tetrahymena patula* during growth in axenic culture. *J. Protozool.* **10,** 74–80.

Suhr-Jessen, P. (1987). Constitutive and induced excretion of polypeptides in *Tetrahymena thermophila*. *J. Protozool.* **34,** 54–57.

Szablewski, L., Andreasen, P. H., Tiedtke, A., Florin-Christensen, J., Florin-Christensen, M., and Rasmussen, L. (1991). *Tetrahymena thermophila:* Growth in synthetic nutrient medium in the presence and absence of glucose. *J. Protozool.* **38,** 62–65.

Thompson, G. A., Jr. (1967). Studies of membrane formation in *Tetrahymena pyriformis*. I. Rates of phospholipid biosynthesis. *Biochemistry* **6,** 2015–2022.

Thompson, G. A., Jr., Baugh, L. C., and Walker, L. F. (1974). Nonlethal deciliation of *Tetrahymena* by a local anesthetic and its utility as a tool for studying cilia regeneration. *J. Cell Biol.* **61,** 253–257.

Tiedtke, A., Hünseler, P., Florin-Christensen, J., and Florin-Christensen, M. (1988). Exocytosis, endocytosis and membrane recycling in *Tetrahymena thermophila*. *J. Cell Sci.* **89,** 515–520.

Tokuyasu, K., and Scherbaum, O. H. (1965). Ultrastructure of mucocysts and pellicle of *Tetrahymena pyriformis*. *J. Cell Biol.* **27,** 67–81.

Tollrian, R., and von Elert, E. (1994). Enrichment and purification of *Chaoborus* kairomone from water: Further steps toward its chemical characterization. *Limnol. Oceanogr.* **39,** 788–796.

Trager, W. (1963). Differentiation in protozoa. *J. Protozool.* **10,** 1–6.

Vaudaux, P. (1976). Isolation and identification of specific cortical proteins in *Tetrahymena pyriformis* strain GL. *J. Protozool.* **23,** 458–464.

Vosskühler, C., and Tiedtke, A. (1993). Magnetic separation of phagosomes of defined age from *Tetrahymena thermophila*. *J. Eukaryot. Microbiol.* **40,** 556–562.

Weidenbach, A. L. S., and Thompson, G. A., Jr. (1974). Studies of membrane formation in *Tetrahymena pyriformis*. VIII. On the origin of membranes surrounding food vacuoles. *J. Protozool.* **21,** 745–751.

Whitson, G. L. (1961). A description of *Tetrahymena vorax* strain Tur. *J. Protozool.* **8**(Suppl.), 20.

Williams, N. E. (1960). The polymorphic life history of *Tetrahymena patula*. *J. Protozool.* **7,** 10–17.

Williams, N. E. (1961). Polymorphism in *Tetrahymena vorax*. *J. Protozool.* **8,** 403–410.

Williams, N. E. (1964). Induced division synchrony in *Tetrahymena vorax*. *J. Protozool.* **11,** 230–236.

Williams, N. E., Vaudaux, P. E., and Skriver, L. (1979). Cytoskeletal proteins of the cell surface in *Tetrahymena*. I. Identification and localization of major proteins. *Exp. Cell Res.* **123,** 311–320.

Williams, N. E., Wolfe, J., and Bleyman, L. K. (1980). Long-term maintenance of *Tetrahymena* spp. *J. Protozool.* **27,** 327.

Williams, N. E., Buhse, H. E., Jr., and Smith, M. G. (1984). Protein similarities in the genus *Tetrahymena* and a description of *Tetrahymena leucophrys* n. sp. *J. Protozool.* **31,** 313–321.

Wolpert, L. (1990). Ordered growth. *Nature* **346,** 711–712.

Yang, X., and Ryals, P. E. (1994). Cytodifferentiation in *Tetrahymena vorax* is linked to glycosylphosphatidylinositol-anchored protein assembly. *Biochem. J.* **298,** 697–703.

Mitosis and Motor Proteins in the Filamentous Ascomycete, *Nectria haematococca*, and Some Related Fungi

James R. Aist
Department of Plant Pathology, College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853

Among filamentous fungi, mitosis has been studied in-depth in just a few species. The mitotic apparatuses in the ascomycetous *Fusarium* spp. are the most clearly and readily visualized *in vivo* within this group; fluorescent labeling is unnecessary. This superior cytological tractability has enabled detailed studies and revealing experiments that have led the way toward a more complete understanding of fungal mitosis. Some of the most important discoveries include the role of half-spindles in development of the bipolar spindle, the existence of true kinetochores in fungi, the unorthodox chromosome configurations and movements comprising metaphase and anaphase A, the attachment of astral microtubules to the plasmalemma, the role of the astral pulling force in elongating the spindle, an inwardly directed force within the spindle, and microtubule cross-bridging in both spindle and asters. Recent research has focused on the roles of microtubuleassociated motor proteins in *Fusarium solani* f. sp. *pisi* (anamorph of *Nectria haematococca*). Cytoplasmic dynein was shown to be involved in the development and/or maintenance of mitotic asters and necessary for motility and functionality of the interphase spindle pole body. The inwardly directed force within the anaphase spindle was shown to be produced by a kinesin-related protein, NhKRP1. Because of its superior cytological tractability, the considerable and unique knowledge we have of many aspects of its mitosis, and its genetic tractability, *Fusarium solani* f. sp. *pisi* is a good choice for further investigations of mitosis in filamentous fungi.

KEY WORDS: Anaphase, Aster, Fungi, Mitosis, Motor protein, Mitotic spindle.

I. Introduction

The manner in which somatic fungal nuclei divide and the mechanisms by which fungal mitosis is achieved have been matters of interest, confusion, and controversy to mycologists and cell biologists for more than a century (Aist and Morris, 1999). Because fungi have played a key role in the relatively recent development of the field of molecular biology, great strides have been made in the understanding of fungal mitosis at the molecular level. Among fungi, the budding yeast, *Saccharomyces cerevisiae,* has proven to be a most useful model system for molecular studies (Hildebrandt and Hoyt, 2000; Winey and O'Toole, 2001) and is the fungus most extensively studied at this level. The fission yeast, *Schizosaccharomyces pombe,* is a close second (Su and Yanagida, 1997). However, differences between mitosis in yeasts and filamentous fungi found at both the cellular and the molecular level have led to the conclusion that some of the basic mechanisms of mitosis may be importantly dissimilar in the two groups. For example, spindle ultrastructure, aster structure and function, and the time course of both anaphase A and B are different in yeasts and filamentous fungi. Also, several of the functions of microtubule (MT)-associated motor proteins in the budding yeast have to do with migration and orientation of the spindle with respect to the bud neck (Cottingham *et al.,* 1999), which has no counterpart in the hyphae of filamentous fungi. Therefore, it would seem prudent to be cautious when extrapolating conclusions about yeast mitosis to mitosis in filamentous fungi. Despite the preponderance of truly elegant work on fungal mitosis that has been done with the yeasts, it remains necessary to examine mitosis more closely in the filamentous fungi as well in order to establish how their nuclei divide.

Several filamentous fungi are potential model systems for the study of mitosis at the molecular level, and a few are contributing significantly to our understanding of mitotic mechanisms (Aist and Morris, 1999). *Nectria haematococca* (hereinafter referred to by the name of its anamorph, or asexual state, *Fusarium solani* f. sp. *pisi*) and *Fusarium* anamorphs of allied fungi (along with *Fusarium solani* f. sp. *pisi,* hereinafter referred to collectively as *Fusarium* spp.) are the most cytologically tractable among all of the fungi studied to date. This characteristic has enabled several unique and important observations to be made and experiments to be performed *in vivo* with these species that are not readily feasible or are impossible with other fungi. This research has clarified much of the confusion and controversy that has surrounded the study of mitosis in filamentous fungi because the results have been extrapolated to fill in the gaps where details are more obscure in the cytologically less tractable species. Therefore, it is important to review these research results and their implications as a way to more firmly establish some of the basic aspects of mitosis in filamentous fungi and to see more clearly where future efforts should be aimed and focused. For a detailed and historical account of mitosis in filamentous fungi in general, see the recent review by Aist and Morris

(1999). Although the focus of this review is on *Fusarium* spp., significant and pertinent results obtained with other filamentous fungi and with yeasts will be referenced occasionally to convey a better overall perspective.

II. Description of Mitosis

With the current emphasis of mitosis research on experimental molecular biology, the basic features, structures, architecture, and sequential processes of mitosis can easily be overlooked. When this happens, the foundation and framework for interpreting and understanding the implications of the results of molecular approaches are lost. Therefore, this review begins with an in-depth examination of mitosis in a few, select filamentous fungi (i.e., *Fusarium* spp.) as gleaned from descriptive studies employing both light and electron microscopic approaches. While reading the following sections, it should be helpful to bear in mind several key points. First, the functional, fungal equivalent of the animal centrosome is the spindle pole body (SPB), a variously shaped microtubule-organizing center that is attached to the cytoplasmic face of the nuclear envelope. Second, fungal mitosis, like animal mitosis, is "astral"—that is, during a large portion of the mitotic process, the spindle poles are associated with cytoplasmic microtubules (MTs) that are attached to the SPB and, at least in *Fusarium* spp., are organized into a funnel-shaped array focused at the SPB (Aist and Bayles, 1991a,d). Third, mitosis in living cells of a broad range of filamentous fungi closely resembles that in *Fusarium* spp. (Aist and Morris, 1999). Finally, relatively little research has been reported on the role of the actin–myosin motility system in mitosis in filamentous fungi, presumably because it has not been possible to demonstrate that such a motility system is particularly important. For example, preliminary, but fairly extensive, results with cytochalasin E have failed to reveal any significant effect of this antiactin drug on spindle elongation during anaphase B in *F. solani* f. sp. *pisi* (J. R. Aist, unpublished results). Consequently, little mention will be made of the actin–myosin motility system or its components in this review.

A. Interphase

Interphase nuclei of *Fusarium* spp. are fairly typical of those of the filamentous fungi, with the exception that all the major nuclear components are clearly visible *in vivo* (Fig. 1). There is a nuclear envelope composed of two unit membranes with nuclear pores, and the SPB is attached to the cytoplasmic face of the nuclear envelope (Fig. 2). In chemically fixed cells, the SPB has a diffuse, fluffy appearance (Aist and Williams, 1972), whereas the SPB appears as a dense, multilayered, flat wafer in freeze-substituted cells (Fig. 2). Typically, there are several cytoplasmic

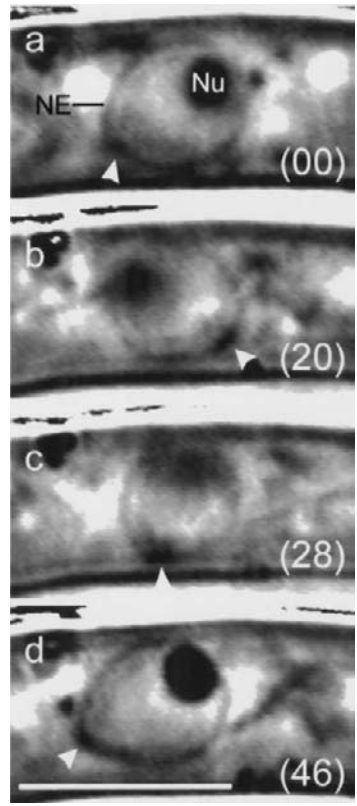


FIG. 1 A phase-contrast, time-lapse series (a–d) of video micrographs showing motility of an SPB at interphase in *Fusarium solani* f. sp. *pisi.* NE, nuclear envelope; Nu, nucleolus. Elapsed time (in seconds) is shown in the lower right corner of each panel. The position of the SPB is indicated by white arrowheads. Scale bar-5 μm (from Inoue *et al.,* 1998a; adapted with permission from Company of Biologists Ltd.)

MTs in close proximity to the interphase SPBs (Fig. 2), and these MTs are usually not in the typical astral configuration that is seen during mitosis. Inside the nucleus is a well-defined nucleolus (Fig. 1).

An accumulation of heterochromatin is attached to the nuclear face of the nuclear envelope subjacent to the SPB (Fig. 2). Based on circumstantial evidence (Aist and Williams, 1972), this heterochromatin has been proposed to include the kinetochores of the interphase chromosomes in *Fusarium oxysporum,* providing a connection between the chromosomes and the SPB throughout interphase, when the chromosomes are not joined to the SPB by kinetochore MTs of the mitotic spindle. There is more direct and recent evidence for such an interphase arrangement in yeasts (Funabiki *et al.,* 1993; Jin *et al.,* 2000; O'Toole *et al.,* 1999). Although

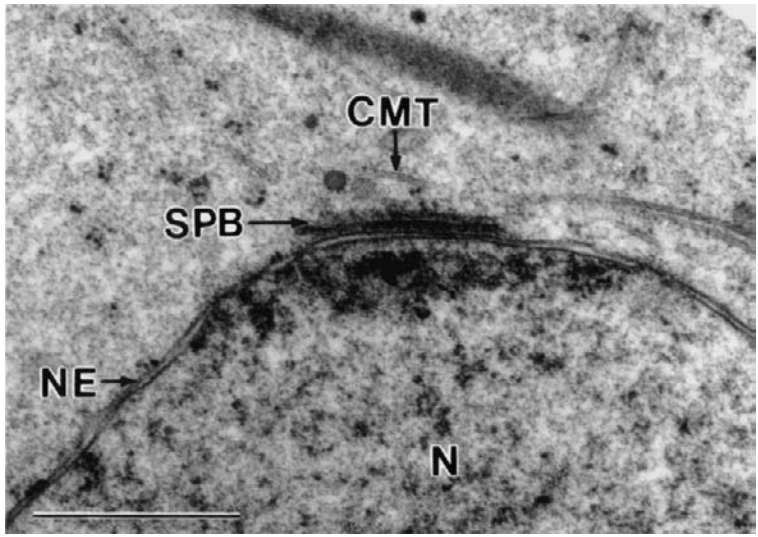


FIG. 2 A transmission electron micrograph of a thin section through a portion of an interphase nucleus (N) in a cell of *Fusarium solani* f. sp. *pisi* that was prepared by freeze substitution. Within the nucleus and subjacent to the nuclear envelope (NE) at the spindle pole body (SPB) is an accumulation of fibrillogranular material composed of heterochromatin and, possibly, kinetochores. Several cytoplasmic microtubules (CMT) are associated with the SPB, which has a layered appearance. Scale bar-1 μm.

Harper (1905) documented the attachments of interphase chromosomes to the SPB in filamentous fungi almost a century ago and first proposed the concept that fungal chromosomes are attached to the SPB, directly or indirectly (i.e., via kinetochore MTs), during the entire fungal life cycle, the biological significance of these attachments is still a matter of speculation. Helpful discussions of progress in the documentation and understanding of this intriguing phenomenon can be found in Aist and Williams (1972), Girbardt (1971), and Jin *et al.* (2000).

By use of phase-contrast or differential interference-contrast optics, the SPB can be visualized easily in living cells of *Fusarium* spp. without the aid and potential pitfalls of a fluorescent label such as green fluorescent protein (GFP) (Fernandez-Abalos, 1998; Xiang *et al.,* 2000). The SPBs are in almost constant motion during interphase (Fig. 1; Inoue *et al.,* 1998a). Motility of the SPB may indent or extend the nuclear envelope momentarily, and nuclei may be translocated a short distance when the SPB pulls momentarily on the nuclear envelope. Long-distance migration of interphase, hyphal nuclei of *Fusarium* spp. appears to be less common than in other filamentous fungi, in which the SPB has been seen to lead the migrating nucleus and pull it to a new position in the cell (Wilson and Aist, 1967). In addition to its role in nuclear motility (discussed in more detail in Section II.F), the SPB of *F. solani* f. sp. *pisi* can also serve as an anchor to hold the interphase nucleus in

place: When the nucleolus was pulled strongly by a laser trap, the SPB apparently resisted the pulling force and kept the nucleus close to its original position in the hypha (Berns *et al.,* 1992). Moreover, when interphase astral MTs were prevented from forming—by disrupting the gene encoding cytoplasmic dynein—the SPBs lost the ability to resist the pull of a laser trap (Inoue *et al.,* 1998a). This result demonstrated a role for astral MTs in the anchoring of interphase nuclei by their SPBs. The roles of SPBs and MTs in nuclear motility of filamentous fungi were reviewed in more detail by Aist (1995).

B. Prophase

In *Fusarium* spp., many of the details of prophase are still to be elucidated. However, the cytological clarity of the nuclei and cytoplasm *in vivo* has enabled several important aspects to be observed and documented with living cells.

As a nucleus is entering prophase, it tends to assume a more spherical shape (Aist, 1969) and becomes brighter in phase contrast. Usually, the nucleolus begins to exhibit Brownian motion, loses some contrast, shrinks in size, and eventually disappears before metaphase (Aist, 1969). The nuclear envelope, however, remains intact. Sister SPBs can be seen to separate and migrate apart in a direction generally perpendicular to the long axis of the hyphal cell, producing two intranuclear, microtubular half-spindles in the process (Aist, 1969; Aist and Morris, 1999). These prophase events were illustrated recently in living cells of *F. solani* f. sp. *pisi* (Fig. 3; Aist and Morris, 1999). Concurrently with the development of the spindle, the condensing chromatin becomes visible as an aggregation of light-gray bodies within and to one side of the nucleus, where the SPBs are separating and generating the half-spindles (Fig. 3; Aist, 1969). Once the half-spindles and condensing chromosomes are apparent, there is no longer an accumulation of hererochromatin subjacent to the SPBs (Aist and Williams, 1972), a possible indication that the kinetochores (KCs) have been carried out into the nucleoplasm by elongation of the KC MTs. By the time the two half-spindles have separated and rotated enough to become parallel and joined into one bipolar spindle, the chromosomes have become clearly differentiated and well contrasted within the nucleoplasm and chromosome arms can be clearly discerned (Fig. 3; Aist and Morris, 1999).

The components of the prophase mitotic apparatus (SPBs, spindle, and chromosomes) now begin to exhibit rocking motions in unison, indicating that the chromosomes are already attached to the spindle by this stage (Aist, 1969). In *F. oxysporum* there are few, if any, cytoplasmic MTs associated with the SPBs during prophase (Aist and Williams, 1972); therefore, it appears that, in this fungus at least, the nuclear movements occurring throughout this phase may not be MT mediated. However, cytoplasmic MTs are associated with the prophase SPBs in the filamentous ascomycete, *Ceratocystis fagacearum* (Aist and Morris, 1999). Why and how the sister SPBs migrate apart perpendicularly to the long axis of

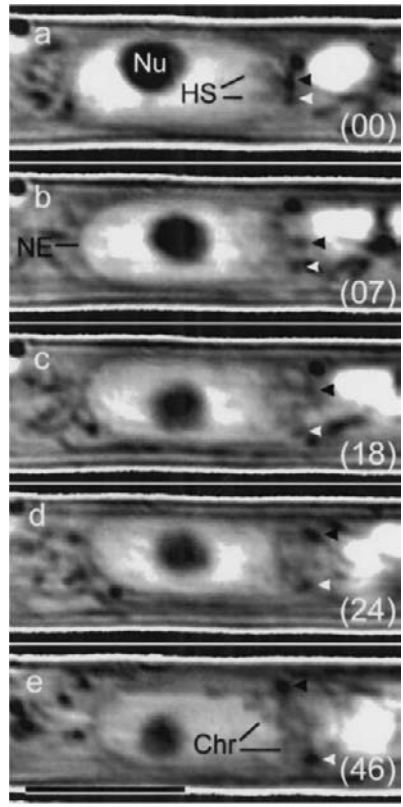


FIG. 3 A time-lapse series (a–e) of video micrographs showing separation of the daughter spindle pole bodies (black and white arrowheads, respectively) during prophase of a mitotic nucleus in *Fusarium solani* f. sp. *pisi,* with the formation of a bipolar spindle. (a) The two SPBs and accompanying half-spindles (HS) are apparent. (b and c) The SPBs separate further along the nuclear envelope (NE). (d and e) chromosome (Chr) condensation can be seen, in association with the developing spindle. Elapsed time (in seconds) is displayed in the lower-right corner of each frame. Nu, nucleolus. Scale bar-5 μm (adapted with permission from Aist and Morris, 1999).

the cell, rather than in a random direction, remains a mystery. Neither *in vivo* nor ultrastructural observations have indicated that the separating sister SPBs are either physically associated with or structurally attached to the cell cortex during this process, although when cytoplasmic (astral) MTs are present at prophase, as in *C. fagacearum,* they could provide such an attachment.

Mitosis in fungi is generally quite rapid relative to that in other eukaryotes (Aist and Williams, 1972). In *F. oxysporum,* prophase takes approximately 70 s, on average, from the first changes in the nucleolus to the beginning of metaphase, when well-contrasted chromosomes are moving in conjunction with a clearly bipolar spindle (Aist and Williams, 1972).

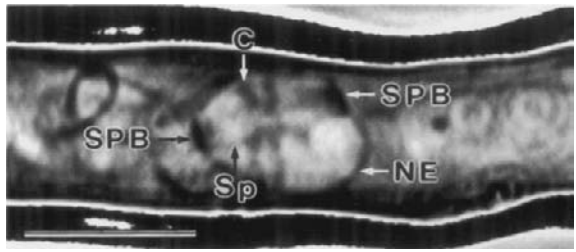


FIG. 4 A phase-contrast video micrograph of metaphase in a living cell of *Fusarium solani* f. sp. *pisi* illustrating the clear visibility of the nuclear envelope (NE), the spindle pole bodies (SPB), the chromosomes (C), and the spindle (Sp). Scale bar-5 μm.

C. Metaphase

Metaphase is a stage during which relatively little morphological change takes place in the mitotic nucleus. Although cytoplasmic (astral) MTs are absent, at least until just before anaphase A begins (Aist and Bayles, 1988, 1991a; Aist and Williams, 1972), metaphase nuclei typically rotate or rock back and forth in place, or they may rotate while migrating over several micrometers to a different location in the cell before entering anaphase (Aist, 1969; Aist and Bayles, 1988). The metaphase spindle of *Fusarium* spp. is visible in living cells (Fig. 4; Aist, 1969; Aist and Bayles, 1988). It is broad in the middle and narrow at the spindle poles, where the spindle MTs converge toward the respective SPBs (Aist and Bayles, 1991b). Most of the spindle MTs are attached to one pole or the other, whereas some are free in the nucleoplasm and a few are continuous from pole to pole (Aist and Bayles, 1991b; Aist and Williams, 1972). The number and total length of spindle MTs are maximal at metaphase (Aist and Bayles, 1991b), and spindle MTs are heavily cross-bridged at this stage (Jensen *et al.,* 1991).

Metaphase chromosomes in *Fusarium* spp. are very well contrasted *in vivo,* appearing dark gray to black in standard preparations viewed with phase-contrast optics (Fig. 4; Aist, 1969; Aist and Bayles, 1988). They are attached to the spindle by minimally differentiated, ball-in-cup KCs that are attached to one MT each (Aist and Bayles, 1991b; Aist and Williams, 1972). In both living and fixed specimens, the chromosomes are not aligned in an equatorial zone to form a typical metaphase plate; rather, they are attached at different points along the central one-half to two-thirds of the spindle (Fig. 4; Aist, 1969; Aist and Bayles, 1991b). In early metaphase, most of the KCs are not arranged in pairs (Aist and Bayles, 1991b), whereas by mid- to late metaphase many of the KCs are paired, but sister KCs are already slightly separated (Aist and Williams, 1972). Such precocious separation of sister KCs was reported also for the fission yeast (Nabeshima *et al.,* 1998).

By late metaphase, the spindle in *Fusarium* spp. has become narrower and elongated (at a rate of 0.6 μm/min in *F. solani* f. sp. *pisi*) by about 1 or 2 μm

(Aist and Bayles, 1988), and the nuclear envelope is still intact (Aist and Berns, 1981; Aist and Williams, 1972). Metaphase lasts about 2 min in *F. oxysporum,* representing a disproportionate amount of the total time in mitosis relative to that of other eukaryotes (Aist and Williams, 1972).

D. Anaphase A

Anaphase A is the dynamic mitotic stage during which the sister chromatids separate further and migrate along the spindle to opposite spindle poles (Inoué and Ritter, 1975). In filamentous fungi, this occurs within a more or less intact nuclear envelope (Aist, 1969; Aist and Berns, 1981; Aist and Williams, 1972, Bayles *et al.,* 1993). The KCs in *F. oxysporum* are found at the spindle poles at the end of anaphase A (Aist and Williams, 1972), which verifies that this stage is functionally equivalent to that in higher eukaryotes. In *Fusarium* spp. it is sometimes possible to observe individual sister chromatids separating to opposite spindle poles in living preparations (Fig. 5; Aist, 1969; Aist and Bayles, 1988). From their different starting points along the middle one-half to two-thirds of the spindle (Fig. 4), the sister chromatids begin their poleward migration asynchronously, creating a momentary mitotic figure in which the chromatids are strung out along most or all of the spindle length, sometimes in two rows, as individual chromatids pass each other on their way to their respective poles (Aist, 1969; Aist and Morris, 1999). This phase has been referred to as the "two-track" or "double-track" stage, and its true identity as anaphase A was not recognized until an accurate

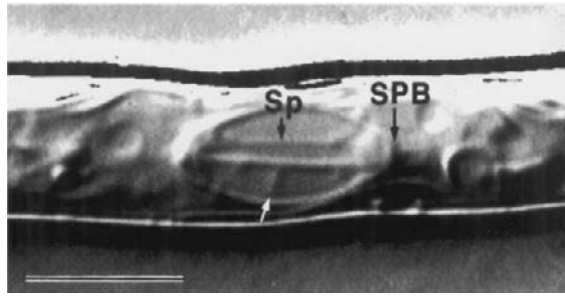


FIG. 5 A differential interference-contrast video micrograph of an anaphase A nucleus in *Fusarium solani* f. sp. *pisi* showing two bundles of spindle microtubules (Sp) comprising the spindle and a chromatid (white arrow) that was migrating toward the spindle pole body (SPB) to the right. The clarity of both the chromatid and the SPB illustrate why it was possible to measure accurately the poleward migration rates of the chromatids in this fungus without the need for fluorescent markers. Scale bar-5 μm (from Aist and Bayles, Video motion analysis of mitotic events in living cells of the fungus *Fusarium solani, Cell Motil. Cytoskel.*).

description of it, as seen in living cells of *F. oxysporum,* was published (Aist, 1969). Individual chromatids of wild-type *Fusarium* spp. are so clearly imaged using differential interference-contrast optics (Fig. 5) that it is possible to measure accurately their rates of migration to the spindle poles at anaphase A with computer-assisted video microscopy techniques (Aist and Bayles, 1988). Different chromatids within a given mitotic nucleus migrate to the spindle pole at different rates. Their migration is typically punctuated by brief moments when the chromatid pauses before completing its journey to the pole. The fastest average rate of anaphase chromatid migration ever recorded for any organism was 7.5 μm/min reported for *F. solani* f. sp. *pisi* (Aist and Bayles, 1988). Anaphase A requires about 13 s in *F. oxysporum* (Aist and Williams, 1972) and 30–45 s in *F. solani* f. sp. *pisi* (Aist and Bayles, 1988). The time for anaphase A in a basidiomycete was about the same as in *F. solani* f. sp. *pisi* (Bayles *et al.,* 1993), and in budding yeast cells the initial poleward movement of chromatids—comprising most of anaphase A—lasts about 25 s (Straight *et al.,* 1997).

During anaphase A, the MTs of the mitotic apparatus undergo significant changes as well. Mitotic asters are developed during this stage (Aist and Bayles, 1988; Aist and Williams, 1972) as MTs are polymerized at the cytoplasmic face of the SPB. The development of asters is correlated with a marked increase in the rate of spindle elongation, from 0.6 μm/min during metaphase to 3.6 μm/min during anaphase A in *F. solani* f. sp. *pisi* (Aist and Bayles, 1988), suggesting that the asters may play a role in the deployment of forces driving spindle elongation (This point will be further discussed later).

In addition to the increase in the rate of spindle elongation in *F. solani* f. sp. *pisi* noted previously, other changes in the spindle occur during anaphase A. Typically, the spindle is composed mainly of two or three bundles of MTs at mid-anaphase A (Fig. 5), but by the end of this phase usually the bundles have been drawn together into one central bundle of MTs (Fig. 6; Aist and Bayles, 1991b; Aist and Berns, 1981). MT cross-bridging occurs in the anaphase A spindle and would be expected to play a role in MT bundling (Jensen *et al.,* 1991). Both the number and the total length of spindle MTs drop precipitously during anaphase A—changes that are too great to be accounted for solely by the depolymerization of KC MTs, which in *F. solani* f. sp. *pisi* would number only about 15 per genome (Aist and Bayles, 1991b). Thus, anaphase A clearly represents a transition phase with respect to mitotic MT dynamics, as intranuclear spindle MTs are depolymerizing while cytoplasmic, astral MTs are polymerizing.

E. Anaphase B

Anaphase B begins when all the chromatids have reached the spindle poles (Inoué and Ritter, 1975), and it ends, by definition (Bayles *et al.,* 1993), when the central spindle has been fully extended and is no longer continuous from pole to pole.

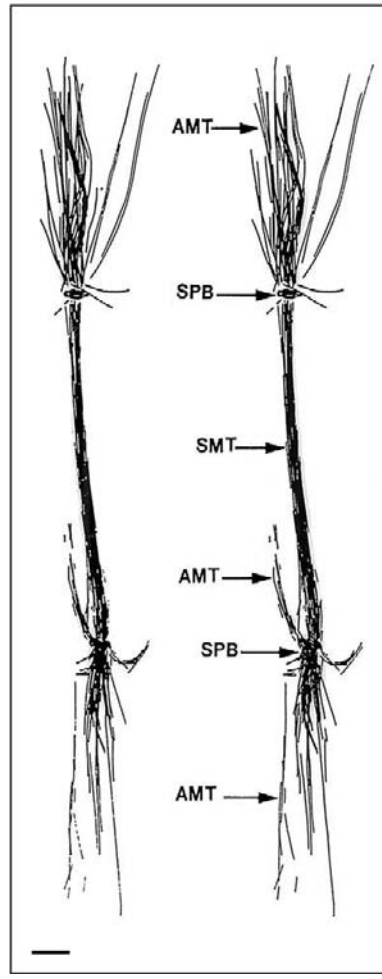


FIG. 6 A three-dimensional stereo-pair reconstruction of the microtubules and spindle pole bodies (SPB) of an entire mitotic apparatus of *Fusarium solani* f. sp. *pisi* at midanaphase B. AMT, astral microtubules; SMT, spindle microtubules. Scale bar-1 μm (reproduced with permission from Jensen *et al.,* 1991).

This is the mitotic stage during which the central spindle elongates rapidly and considerably, up to three or four times its metaphase length in *Fusarium* spp. (Aist, 1969; Aist and Bayles, 1988). In *F. solani* f. sp. *pisi,* the rate of spindle elongation (as measured in living cells) is an almost unprecedented 6 or 7 μm/min (Aist and Bayles, 1988). By comparison, the rate in fission yeast is 1.4 or 1.5 μm/min (Hagan *et al.,* 1990; Nabeshima *et al.,* 1998) and in budding yeast the fast phase occurs at nearly the same rate as in fission yeast (Kahana *et al.,* 1995; Saunders *et al.,*1995;

Yeh *et al.,* 1995). Anaphase B takes slightly more than 1 min in *F. oxysporum* (Aist and Williams, 1972) and about 2 min in *F. solani* f. sp. *pisi* (Aist and Bayles, 1988). Typically, the anaphase B nucleus in *Fusarium* spp. oscillates back and forth within the cell (Aist, 1969; Aist and Bayles, 1988), which was one of the first indications that the mitotic apparatus may be pulled on during this stage by an extranuclear force acting on the SPBs via astral MTs.

The nuclear envelope becomes greatly attenuated during anaphase B by forces that elongate the spindle because it does not break down until late in this stage (Aist, 1969; Aist and Berns, 1981; Aist and Williams, 1972). Laser trapping experiments (Berns *et al.,* 1992) have demonstrated the amazing elasticity of the nuclear envelope in living cells of *F. solani* f. sp. *pisi.* As the spindle is elongated, the subterminal portions of the original, now attenuated, nuclear envelope pinch down behind the incipient daughter nuclei (Aist, 1969; Aist and Berns, 1981; Aist and Williams, 1972); thus, the nuclear envelopes surrounding the daughter nuclei are derived directly from the corresponding ends of the original nuclear envelope during anaphase B. In late anaphase B, the remnant nuclear envelope between the daughter nuclei disintegrates and disappears from view in living cells (Aist, 1969; Aist and Bayles, 1988).

Only recently have mitotic asters garnered much attention in the search for mitotic mechanisms in any major group of organisms. Even after it was established that mitotic fungal nuclei have fairly well-developed asters, almost everyone assumed that forces developed within the spindle were solely responsible for separation of the daughter genomes during anaphase B (Aist and Morris, 1999). Consequently, most of the descriptive research on mitosis in fungi virtually ignored the mitotic asters, even until the mid-1990s (Ding *et al.,* 1993; Winey *et al.,* 1995). Documentation of the mitotic asters in *Fusarium* spp. began in the early 1970s when Aist and Williams (1972) reported that the mitotic apparatus in chemically fixed hyphae of *F. oxysporum* included microtubular asters that were developing during anaphase A, became maximally developed during anaphase B, and diminished to the minimal interphase state during telophase.

Mitotic asters of *F. solani* f. sp. *pisi* have been extensively documented and analyzed in living cells (Aist and Bayles, 1991d) and in cells prepared by freeze substitution (Aist and Bayles, 1991a), a method that greatly improves the preservation of organelles, especially the MTs (Howard and Aist, 1979; Howard and O'Donnell, 1987). In living cells, the asters cannot be seen directly, but directed movements of organelles within the asters toward and from the SPB reveal the shape and extent of the astral region (Aist and Bayles, 1991d). The movements of organelles in the astral region occur about equally in both directions, such that little or no buildup of organelles occurs in the aster, in contrast to the marked accumulation of organelles in asters of animal cells (Rebhun, 1972) and in those of a basidiomycete fungus (Girbardt, 1968). Electron microscopy has provided evidence that membrane-bounded organelles within the asters of *F. solani* f. sp. *pisi* are cross-bridged to the astral MTs (Aist and Bayles, 1991d). Such cross-bridging could represent the structural manifestation of the MT-associated motor

proteins that would be expected to generate the mechanical force to move the organelles within the asters. Moreover, it is possible that a mechanical force that would draw the tip of a mitochondrion toward the SPB at anaphase B would be offset by a corresponding mechanical force in the opposite direction since the entire mitochondria do not get pulled into the aster but rather seem to simply become stretched out in the direction of the SPB and then spring back suddenly in the opposite direction (Aist and Bayles, 1991d). In such a case, this tugging on mitochondria could contribute to the astral pulling force during anaphase B, especially if the mitochondria were anchored to cytoskeletal structures, such as F-actin, intermediate filaments, or cytoplasmic MTs.

Ultrastructural analyses (Aist and Bayles, 1991a; Jensen *et al.,* 1991) of asters in *F. solani* f. sp. *pisi* provided a wealth of new structural information. The MTs of these asters are of three types: attached to the SPB, not attached to anything, and not attached to the SPB but likely cross-bridged to other MTs that are attached. Many of the astral MTs were shown to be cross-bridged to other astral MTs. The deployment of MTs in the mitotic asters gave the impression that the MTs were exhibiting dynamic instability and were in a state of tension which pulled them from their moorings at the cytoplasmic surface of the SPB. The two asters of an anaphase B nucleus may be of different sizes at any given moment, which could explain how the astral pulling force could fluctuate and produce the oscillations of the entire mitotic apparatus that are characteristic of this stage (Aist and Bayles, 1988). Putative cross-bridges between astral MTs were reported to occur also in the mitotic apparatus of a basidiomycete (O'Donnell, 1994).

Finally, the distal tips of a few of the astral MTs were shown, as early as 1981, to be associated with the plasma membrane via a localized, fibrillogranular, flocculent material located in the cell cortex subjacent to the plasma membrane at the point of astral MT termination (Fig. 7; Aist and Bayles, 1991a; Aist and Berns, 1981). Other astral MTs were associated laterally with linear elements in the astral regions that were of the diameter and appearance of intermediate filaments (Aist and Bayles, 1991a). Either or both of these astral MT associations could theoretically contribute to the astral pulling force. Many recent studies of mitotic asters in fungi have dealt with the relationships of mitotic asters with the plasma membrane in yeasts (Goode *et al.,* 2000; Heil-Chapdelaine *et al.,* 1999) and a basidiomycete (O'Donnell, 1994).

The inference that mitotic spindles in filamentous ascomycetes develop within them an outwardly directed force (herein called the "spindle pushing force") that pushes against the SPBs and aids in their separation (i.e., spindle elongation) during anaphase was based on the evidence of a few illustrations of bent spindles, several of which were published about a century ago (Aist and Morris, 1999). Recently, precise measurements of spindle length during episodes of spindle bending in living cells of *F. solani* f. sp. *pisi* (Aist and Bayles, 1991c) demonstrated that spindle bending is fairly common but fleeting, and that anaphase spindles bend because of pushing forces that are developed within them. This finding was concordant and virtually contemporary with the earliest discoveries that MT-associated motor proteins in the spindles of other fungi are involved in the production of

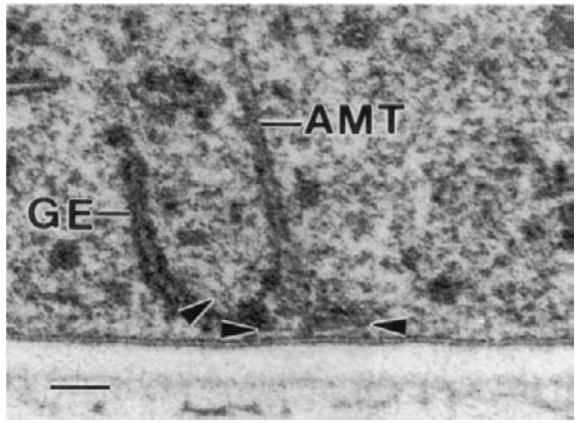


FIG. 7 An electron micrograph showing the plus end of an astral microtubule (AMT) of an anaphase B nucleus of *Fusarium solani* f. sp. *pisi* embedded in a tuft of thin filaments (arrowheads) in the cell cortex, subjacent to the plasma membrane. GE, Golgi equivalent. Scale bar-0.1 μm (from Aist and Bayles, 1991a).

force for spindle elongation (Enos and Morris, 1990; Hagan and Yanagida, 1990; Meluh and Rose, 1990).

During anaphase B in *F. solani* f. sp. *pisi,* the shortest MTs were selectively depolymerized without evidence of any MT growth taking place in the spindle (Aist and Bayles, 1991b). Coupled with the extensive lengthening of the spindle at this stage, this depolymerization led to a visible reduction in the diameter of the spindle during anaphase B, which was reflected in a much smaller number of spindle MT profiles in an average cross section (Jensen *et al.,* 1991). No continuous, pole-to-pole MTs were found in the anaphase B spindle, but the MT fragments comprising the spindle were heavily cross-bridged (Jensen *et al.,* 1991), apparently preserving the integrity of the spindle as well as its force-generating capability. The overall results of detailed, fine-structural analyses of MTs making up the spindle of *F. solani* f. sp. *pisi* (Fig. 6; Aist and Bayles, 1991b; Jensen *et al.,* 1991)—especially the lack of a central, overlap zone of antiparallel MTs—led to the development of a "telescoping" model for spindle elongation in which the spindle would be elongated primarily by the astral pulling force much like one would extend a telescope. According to this model, the MT fragments comprising the spindle would be slid passively past each other, giving rise to both spindle elongation and a narrowing of the spindle during anaphase B, as was observed (Aist and Bayles, 1991b).

F. Telophase

The main events of telophase include a reappearance and enlargement of the nucleolus, enlargement of the daughter nuclei to their interphase size, decondensation of

the chromatin resulting in a brighter appearance of the nuclei with phase-contrast optics, and a period of rapid, postmitotic nuclear migration during which the daughter nuclei become positioned prior to septum formation (Aist, 1969, 1995). Although the natural breaking of the spindle is used to define the onset of telophase (Bayles *et al.,* 1993), telophase events involving the nucleolus, the chromatin, and nuclear size frequently begin moments before the spindle breaks. Thus, there is sometimes overlap between the anaphase B and telophase stages regarding the behavior of the various nuclear components. This is one reason why it is helpful to use only one of several available criteria, (i.e., spindle breakdown) to define the starting point for telophase. The other reason is that the daughter nuclei are not truly independent of each other until spindle breakdown; therefore, technically, the nucleus is still dividing.

III. Experimental Studies of Mitotic Forces

Until about 1980, little was known about mitotic forces that elongate the spindle in any fungus, although many speculations had been published. It was assumed that the spindle elongated under its own power, and that the mitotic asters were not involved (Aist and Morris, 1999). This delegation of anaphase mitotic function to the spindle exclusively began to change radically with the publication of a laser microbeam (Berns *et al.,* 1981) study in the early 1980s (Aist and Berns, 1981). Nuclei of *F. solani* f. sp. *pisi* at early anaphase B were precisely targeted *in vivo* for irradiation at the central spindle, effectively cutting the spindle and neutralizing any force that may reside in it. If, in fact, the spindle were elongating under its own power alone, cutting the spindle would prevent the SPBs from further separating from each other during the remainder of anaphase B. However, the opposite result was obtained: When the spindle was cut, the SPBs separated from each other at about three times the rate observed when the spindle was left intact in control cells. This surprising, but clear-cut and reproducible, result necessitated a rethinking of the forces that are at work during anaphase B. It was obvious that an extranuclear pulling force was being applied to the SPBs, presumably by the asters. This result provided the first evidence that fungi have redundant mechanisms—spindle pushing and astral pulling—to elongate their spindles. The results revealed also that in the intact spindle, an inwardly directed counterforce was resisting spindle elongation and opposing the pulling force. Thus, both an astral pulling force and an inwardly directed spindle counterforce were demonstrated in the same fungus by this experiment. Many subsequent studies have confirmed this conclusion and revealed the source of these forces to be MT-associated motor proteins (Aist and Morris, 1999; Hildebrandt and Hoyt, 2000; Su and Yanagida, 1997). Moreover, a 1991 study of spindle bending (Aist and Bayles, 1991c), coupled with the earlier laser microbeam study (Aist and Berns, 1981), established *F. solani* f. sp. *pisi* as the only fungus in which all three mitotic forces—spindle pushing, astral pulling, and

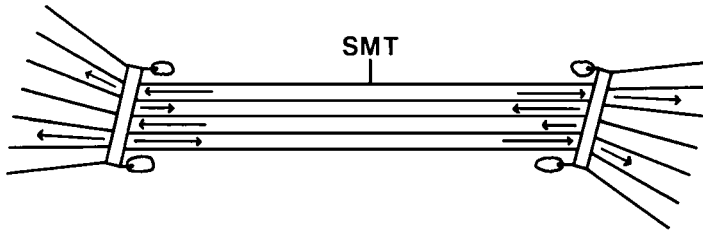


FIG. 8 A diagrammatic representation (not to scale) of forces postulated in 1981 to act on the spindle pole bodies (rectangles) during anaphase B in *Fusarium solani* f. sp. *pisi.* The numbers and lengths of the arrows were intended to represent rough approximations of the magnitudes and directions of the astral pulling force, the spindle pushing force, and the inwardly directed force within the spindle which counteracts the other two forces. Evidence for this model was derived from a unique laser microbeam experiment, as described in the text. SMT, spindle microtubules (reproduced from *The Journal of Cell Biology,* 1981, Vol. **91,** pp. 446–458 by copyright permission of The Rockefeller University Press).

an inwardly directed spindle counterforce—had been identified and demonstrated up to that time. These three mitotic forces were depicted diagrammatically in 1981 in a model for anaphase B in *F. solani* f. sp. *pisi* (Fig. 8; Aist and Berns, 1981). The early laser microbeam studies with *F. solani* f. sp. *pisi* were extended to a filamentous basidiomycete about 12 years later, with similar results and conclusions regarding spindle and aster forces (Bayles *et al.,* 1993). Thus, it appears that these same mitotic forces may be at work in filamentous fungi in general.

The initial laser microbeam experiments with *F. solani* f. sp. *pisi* (Aist and Berns, 1981) left unsettled three important questions regarding deployment of the pulling force: Does this force reside within the astral region? Is this force transmitted via MTs? Does this force aid in the elongation of the anaphase B spindle? All three questions were answered in a follow-up study published a decade later (Aist *et al.,* 1991). When a UV laser microbeam was used to directly irradiate the astral region close to one of the two SPBs at anaphase B, the pulling force in that aster was apparently neutralized as the entire mitotic apparatus migrated rapidly in the opposite direction, presumably being pulled by the (now unopposed) nonirradiated aster at the other spindle pole. This result demonstrated that the pulling force resides in the aster. Cutting the central spindle with a laser microbeam and depolymerizing all MTs in the irradiated cell with the anti-MT agent MBC stopped the SPBs (with their attached incipient daughter nuclei) dead in their tracks, indicating that the pulling force was transmitted via the astral MTs in nontreated cells. When only one of the bundles of spindle MTs was cut by a laser microbeam at very early anaphase B, the elongation rate of the remaining portion of the intact spindle was often increased. This result showed that the astral pulling force can affect the rate of spindle elongation since the opposite result would be expected if the spindle were elongating by virtue of only the spindle pushing force.

One important new concept arising from these studies is that rather than being under compression during anaphase B, the spindle is actually under tension from the astral pulling force almost constantly from the onset of anaphase A until the spindle breaks at late anaphase B (Aist and Berns, 1981; Aist and Bayles, 1991c). The experimental, laser microbeam evidence for this concept in *F. solani* f. sp. *pisi* is corroborated by observations made previously on nonirradiated cells of other fungi: in both the ascomycete, *C. fagacearum,* and the basidiomycete, *Helicobasidium mompa,* the natural breaking of the spindle at the end of anaphase B immediately precedes a rapid increase in the rate at which the SPBs separate from each other (Aist, 1969; Bayles *et al.,* 1993). Thus, the earlier idea (alluded to previously) that only the pushing force developed within the spindle is involved in spindle elongation during anaphase B has long since been thoroughly disproven with respect to mitosis in filamentous fungi.

Subsequent to publication of the laser microbeam studies with *F. solani* f. sp. *pisi,* the concept of an astral pulling force operating during anaphase B gained widespread acceptance and application (Hoyt and Geiser, 1996; McIntosh and Pfarr, 1991; Saunders, 1993). Recent results with the budding yeast have shown that pulling by astral MTs helps to orient and position the mitotic spindle (Carminati and Stearns, 1997; Shaw *et al.,* 1997). Furthermore, as was the case with the astral MTs of *F. solani* f. sp. *pisi* (Aist and Berns, 1981), the pulling force transmitted via the astral MTs can produce a velocity of SPB movement that far exceeds the rate of anaphase B spindle elongation (Yeh *et al.,* 2000).

Other results with yeasts indicate that astral MTs may push, rather than pull, on the SPBs in mitotic cells (Ding *et al.,* 1998; Shaw *et al.,* 1997). Since some of the astral MTs in *F. solani* f. sp. *pisi* are backwardly oriented during anaphase B (Aist and Berns, 1981; Aist and Bayles, 1991a), it would be worthwhile to determine whether the astral force in this fungus is pushing rather than pulling the SPBs.

Perhaps mitotic astral MTs can both push and pull on the SPBs. Astral MTs in mating cells of the budding yeast appear to both push and pull on the SPBs as cortically tethered MTs undergo polymerization and depolymerization, respectively (Maddox *et al.,* 1999). Furthermore, both pulling and pushing of the mitotic spindle poles by astral MTs of the budding yeast have been inferred recently (Yeh *et al.,* 2000).

IV. Motor Proteins in Mitosis

The descriptive and experimental research on mitosis in *F. solani* f. sp. *pisi* (reviewed above) set the stage for a new direction in the research aimed at determining the possible roles of MT-associated motor proteins (Sharp *et al.,* 2000) in generating the mitotic forces that had been identified. By the early 1990s, it had been

demonstrated that (i) mitotic asters are well developed and exert a pulling force on the SPBs that helps to elongate the spindle; (ii) the narrow, central spindle that is elongated between the SPBs during anaphase B generates an inwardly directed counterforce that opposes the astral pulling force, helping to regulate the rate at which the astral pulling force is allowed to elongate the spindle; and (iii) the spindle also produces an outwardly directed pushing force that can elongate the anaphase B spindle at a reduced rate at brief times during which the astral force is momentarily absent or ineffectual.

Since we had determined the locations and directions of the three main mitotic forces at work during anaphase B in *F. solani* f. sp. *pisi* the next logical step was to begin to identify what produces those forces. Because research in other laboratories had begun to show that in the fungi such forces are generated by various MT-associated motor proteins (Enos and Morris, 1990; Hagan and Yanagida, 1990; Meluh and Rose, 1990), we embarked on a mission to identify similar motor proteins in *F. solani* f. sp. *pisi* and determine which, if any, are responsible for the previously demonstrated mitotic forces.

The first such motor protein that we found is the one that is most abundant in *F. solani* f. sp. *pisi,* and sequence analysis revealed it to be a conventional kinesin (Wu *et al.,* 1998). This motor protein has a 77% identity to a corresponding conventional kinesin in *Neurospora crassa.* Moreover, when substituted for the *N. crassa* conventional kinesin gene (*NcKIN*), the *F. solani* f. sp. *pisi* gene (*NhKIN1*) supported a growth rate that was 80% of the wild-type growth rate in *N. crassa* (Kirchner *et al.,* 1999).

Using primers corresponding to conserved regions of known kinesin-encoding genes, we cloned *NhKIN1* from genomic *F. solani* f. sp. *pisi* DNA by polymerase chain reaction amplification (Wu *et al.,* 1998). When we deleted this gene by transformation-mediated homologous recombination, the mutant exhibited several dramatic phenotypes, including a marked reduction in growth rate, smaller hyphae that grew in a helical or wavy manner, withdrawal of the mitochondria from the growing hyphal tips, reduction in the size of the Spitzenkörper (an aggregate of secretory vesicles at the growing tip), and cyclical variations in the position of the Spitzenkörper within the hyphal apex. In concordance with results obtained with other conventional kinesins, there was no significant difference in the rate of anaphase B spindle elongation between the mutant and wild type. Conventional kinesins have a primary role in organelle motility rather than in mitosis (Barton and Goldstein, 1996; Brady, 1995), as our results demonstrated. Although we found no mitotic phenotype in the mutant, the results demonstrated the role of the MT-based motility system in apical transport of secretory vesicles and mitochondria, as well as its role in positioning of the Spitzenkörper. The latter role explains the aberrant hyphal morphology in the mutant since the shifting position of the Spitzenkörper preceded corresponding shifts in growth direction (Wu *et al.,* 1998).

The next MT-associated motor protein that we identified in *F. solani* f. sp. *pisi* was cytoplasmic dynein (Inoue *et al.,* 1998a). Sequencing of the gene *DHC1*

showed that this motor is typical of cytoplasmic dynein heavy chains. It is 78% identical to the corresponding motor in *N. crassa* and 70.2% identical to that in *Aspergillus nidulans,* but only 24.8% identical to that in *Saccharomyces cerevisiae.* By inserting a selectable marker into the central motor domain of *DHC1,* we disrupted the native gene. In addition to a drastic reduction in the growth rate and dramatic effects on hyphal morphogenesis, the mutant exhibited several nuclear phenotypes: Mitotic and postmitotic aster-like arrays of cytoplasmic MTs (i.e., asters) were missing or greatly reduced, postmitotic nuclei failed to migrate normally, SPBs were nonmotile at interphase and did not anchor interphase nuclei as in wild type, and there was a nonuniform distribution of interphase nuclei (Inoue *et al.,* 1998a). Although it was already known that cytoplasmic dynein is involved in nuclear migration in fungi and that dynein mutants exhibit abnormal nuclear migration and/or distribution (Bruno *et al.,* 1996; Li *et al.,* 1993; Plamann *et al.,* 1994; Xiang *et al.,* 1994; Yeh *et al.,* 1995), results with the *F. solani* f. sp. *pisi* dynein mutant relating to a role for dynein in mitotic aster formation and the loss of SPB functions at interphase made unique contributions to our knowledge of the functions of cytoplasmic dynein.

A further examination of the dynein mutant (Inoue *et al.,* 1998b) revealed a subtle mitotic phenotype: The rate and extent of spindle elongation during anaphase B were both less in the mutant than in the SO1–2 (control) isolate (Fig. 9). Moreover, when the spindle in the mutant was cut by a laser microbeam at early anaphase B, SPB separation almost stopped, in contrast to a rapid increase in the SPB separation rate when the SO1–2 (control) spindle was cut (Fig. 9). This result demonstrated unequivocally that no pulling force was present in the mitotic apparatus of the mutant, possibly because the mutant had little or no mitotic aster. Whereas earlier studies of the role of dynein in fungal mitosis had concluded that there is no mitotic phenotype per se in dynein mutants (Li *et al.,* 1993; Xiang *et al.,* 1994), our results demonstrated that, at least in our *F. solani* f. sp. *pisi* mutant, there is a mitotic phenotype that becomes apparent when the time course of mitosis is examined and quantified carefully in living cells. *In vivo* time course studies with dynein mutants of *A. nidulans* (Babcock *et al.,* 1997) and budding yeast (Saunders *et al.,* 1995; Yeh *et al.,* 1995) have reached similar conclusions. Our results with the cytoplasmic dynein mutant of *F. solani* f. sp. *pisi* (Inoue *et al.,* 1998b) confirmed the presence of the spindle pushing force in living cells (previously inferred only from a time course study) because the spindle elongated in the absence of asters. They also showed that the rapid increase in the spindle elongation rate that occurs in the transition from anaphase A to anaphase B (Aist and Bayles, 1998) is due primarily to the development of the astral pulling force, concomitant with the growth of the mitotic asters that occurs at this transition.

In addition to the conventional kinesin mentioned previously, we have also succeeded in cloning and sequencing genes encoding two kinesin-related motor proteins. One of these motor proteins, NhKRP1, is a homolog of the KLPA motor, which produces an inwardly directed force in the mitotic spindle of *A. nidulans*

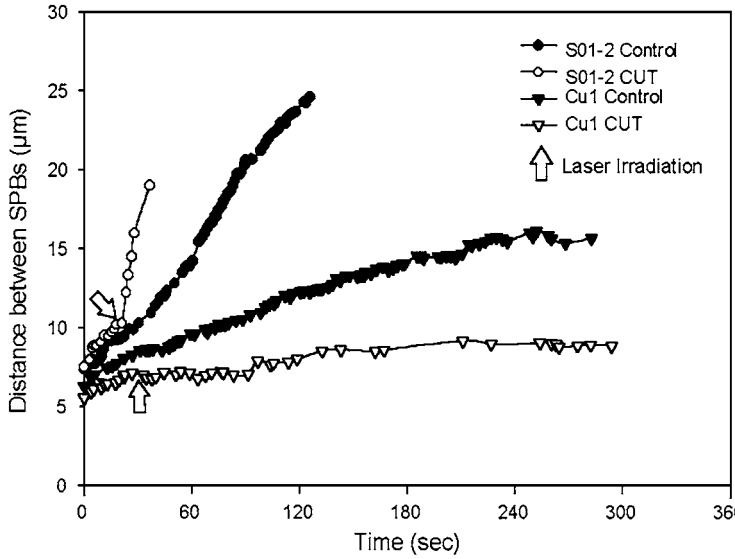


FIG. 9 Plots of spindle pole body separation of the dynein mutant, Cu1, and the ectopic transformant control, SO1–2, following laser microbeam cutting of the spindle (CUT) or comparable control irradiation of the nucleoplasm (control) at early anaphase B in *Fusarium solani* f. sp. *pisi.* Note that spindle pole body separation was accelerated two- or threefold in the SO1–2 control (which had a normal complement of astral microtubules) when the spindle was cut. In contrast, spindle pole body separation almost stopped when the spindle was cut in CU1, demonstrating the absence of the astral pulling force in the dynein mutant (which had few or no astral microtubules). The figure shows also that without astral microtubules (Cu1 control), the spindle elongates at a fraction of the normal rate of spindle elongation (SO1–2 control), reflecting the contribution of the astral pulling force to spindle elongation in this fungus (from Inoue *et al.,* 1998b; reproduced with permission of Company of Biologists Ltd.).

(O'Connell *et al.,* 1993). The other, encoded by the gene *NhKRP2* (GenBank accession No. AF102993), is a homolog of the BIMC motor, which produces an outwardly directed force in the spindle of *A. nidulans* that functions to separate the SPBs during prophase and produce the bipolar spindle (Enos and Morris, 1990). We have succeeded in deleting the *NhKRP1* gene (GenBank accession No. AF102992) in *F. solani* f. sp. *pisi* and determining the mitotic phenotype of the mutant *in vivo* (Sandrock *et al.,* 1999). In the mutant, the rate of spindle elongation during anaphase A was increased by about 50% relative to that in the wild type, whereas the rate during anaphase B was increased about 25%—both highly significant statistical differences. These results are consistent with the expectation that the absence of the inwardly directed force in the spindle of the mutant would allow the spindle to be elongated by the astral pulling forces more rapidly than in wild type. Moreover, they demonstrate once again that a motor protein mutant

that is initially reported to have no mitotic phenotype (O'Connell *et al.,* 1993) may have a demonstrable mitotic phenotype when examined appropriately *in vivo.*

V. Concluding Remarks

The research on *Fusarium* spp. in general, and on *F. solani* f. sp. *pisi* in particular, has established *N. haematococca* (the holomorph of *F. solani* f. sp. *pisi)* as an attractive model system for the study of mitosis in filamentous fungi. As noted above, *Fusarium* spp. as a group stand out among filamentous fungi in having the most easily visualized mitotic apparatus in living cells. Within this group, *F. solani* f. sp. *pisi* is preferred for mitotic studies because its nuclei are somewhat larger than are those of other *Fusarium* spp., its tip cells are predominantly uninucleate (making it more suitable than multinucleate species for laser microbeam experiments), and its cytoplasm affords greater contrast of nuclear components *in vivo* than is achievable with other *Fusarium* spp. The detailed and often unique, basic information on the structure, timing, and forces of mitosis as visualized in both living and fixed cells, when coupled with the considerable genetic and cytological tractability of *F. solani* f. sp. *pisi,* should make it easier to correctly quantify and interpret the significance of mitotic phenotypes in motor protein mutants of this fungus than in those of other filamentous fungi. This has proven to be the case with the cytoplasmic dynein and *krp1* mutants, as discussed above.

One specific goal of future research with *F. solani* f. sp. *pisi* is to generate GFP transformants to study the distribution and behavior of MTs and the localization of motor proteins in living cells. Efforts to obtain a genetically suitable *krp2* mutant and a cytoplasmic dynein mutant that has the full complement of astral MTs should also be a high priority. The latter would enable us to find out whether or not cytoplasmic dynein produces the astral pulling force for mitosis, which we suspect it does. The fact that *F. solani* f. sp. *pisi* is the only cytologically tractable filamentous fungus known in which certain critical observations and experiments can be performed *in vivo* without prior transformations to label components of the mitotic apparatus confers upon this continuing research a unique and valuable importance. Results and conclusions from the other, less cytologically tractable, fungi currently being studied should be tested and confirmed in a fungus with which so much can be achieved in living, wild-type cells. It is important also to find out to what extent the results and conclusions from related research with yeasts actually apply to filamentous fungi, and that should be ascertainable from corresponding results with *F. solani* f. sp. *pisi.*

Acknowledgment

Portions of the reviewed research were supported by National Science Foundation grants for research, including grant number MCB-9807829.

References

Aist, J. R. (1969). The mitotic apparatus in fungi, *Ceratocystis fagacearum* and *Fusarium oxysporum. J. Cell Biol.* **40,** 120–135.

Aist, J. R. (1995). Independent nuclear motility and hyphal tip growth. *Can. J. Bot.* **73,** S122–S125.

Aist, J. R., and Bayles, C. J. (1988). Video motion analysis of mitotic events in living cells of the fungus *Fusarium solani. Cell Motil. Cytoskel.* **9,** 325–336.

Aist, J. R., and Bayles, C. J. (1991a). Ulrastructural basis of mitosis in the fungus Nectria haematococca (sexual stage of *Fusarium solani*). I. Asters. *Protoplasma* **161,** 111–122.

Aist, J. R., and Bayles, C. J. (1991b). Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). II. Spindles. *Protoplasma* **161,** 123–136.

Aist, J. R., and Bayles, C. J. (1991c). Detection of spindle pushing forces in vivo during anaphase B in the fungus *Nectria haematococca. Cell Motil. Cytoskel.* **19,** 18–24.

Aist, J. R., and Bayles, C. J. (1991d). Organelle motility within mitotic asters of the fungus *Nectria haematococca. Eur. J. Cell Biol.* **56,** 358–363.

Aist, J. R., and Berns, M. W. (1981). Mechanics of chromosome separation during mitosis in *Fusarium* (Fungi Imperfecti): New evidence from ultrastructural and laser microbeam experiments. *J. Cell Biol.* **91,** 446–458.

Aist, J. R., and Morris, N. R. (1999). Mitosis in filamentous fungi: How we got where we are. *Fungal Genet. Biol.* **27,** 1–25.

Aist, J. R., and Williams, P. H. (1972). Ultrastructure and time course of mitosis in the fungus *Fusarium oxysporum. J. Cell Biol.* **55,** 368–389.

Aist, J. R., Bayles, C. J., Tao, W., and Berns, M. W. (1991). Direct experimental evidence for the existence, structural basis, and function of astral forces during anaphase B *in vivo. J. Cell Sci.* **100,** 279–288.

Babcock, J. F., Aist, J. R., and Morris, N. R. (1997). Cytoplasmic dynein performs multiple functions in *Aspergillus nidulans. Inoculum* **48,** 2.

Barton, N. R., and Goldstein, L. S. B. (1996). Going mobile: Microtubule motors and chromosome segregation. *Proc. Natl. Acad. Sci. USA* **93,** 1735–1742.

Bayles, C. J., Aist, J. R., and Berns, M. W. (1993). The mechanics of anaphase B in a basidiomycete as revealed by laser microbeam microsurgery. *Exp. Mycol.* **17,** 191–199.

Berns, M. W., Aist, J., Edwards, J., Strahs, K., Girton, J., McNeill, P., Rattner, J. B., Kitzes, M., Hammer-Wilson, M., Liaw, L.-H., Siemens, A., Koonce, M., Peterson, S., Brenner, S., Burt, J., Walter, R., Bryant, P. J., van Dyk, D., Coulombe, J., Cahill, T., and Berns, G. S. (1981). Laser microsurgery in cell and developmental biology. *Science* **213,** 505–513.

Berns, M. W., Aist, J. R., Wright, W. H., and Liang, H. (1992). Optical trapping in animal and fungal cells using a tunable, near-infrared titanium-sapphire laser. *Exp. Cell Res.* **198,** 375–378.

Brady, S. T. (1995). A kinesin medley: Biochemical and functional heterogeneity. *Trends Cell Biol.* **5,** 159–164.

Bruno, K. S., Tinsley, J. H., Minke, P. F., and Plamann, M. (1996). Genetic interactions among cytoplasmic dynein, dynactin, and nuclear distribution mutants of *Neurospora crassa. Proc. Natl. Acad. Sci. USA* **93,** 4775–4780.

Carminati, J. L., and Stearns, T. (1997). Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. *J. Cell Biol.* **138,** 629–641.

Cottingham, F. R., Gheber, L., Miller, D. L., and Hoyt, M. A. (1999). Novel roles for *Saccharomyces cerevisiae* mitotic spindle motors. *J. Cell Biol.* **147,** 335–350.

Ding, R., McDonald, K. L., and McIntosh, J. R. (1993). Three-dimensional reconstruction and analysis of mitotic spindles from the yeast, *Schizosaccharomyces pombe. J. Cell Biol.* **120,** 141–151.

Ding, D. Q., Chikashige, Y., Haraguchi, Y., and Hiraoka, Y. (1998). Oscillatory nuclear movement in fission yeast meiotic prophase is driven by astral microtubules, as revealed by continuous observation of chromosomes and microtubules in living cells. *J. Cell Sci.* **111,** 701–712.

Enos, A. P., and Morris, N. R. (1990). Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in *A. nidulans. Cell* **60,** 1019–1027.

Fernandez-Abalos, J. M., Fox, H., Pitt, C., Wells, B., and Doonan, J. H. (1998). Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans. Mol. Microbiol.* **27,** 121–130.

Funabiki, H., Hagan, I., Uzawa, S., and Yanagida, M. (1993). Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol.* **121,** 961–976.

Girbardt, M. (1968). Ultrastructure and dynamics of the moving nucleus. *In* "Aspects of Cell Motility" (P. L. Miller, Ed.), pp. 249–259. Cambridge Univ. Press, Cambridge,UK.

Girbardt, M. (1971). Ultrastructure of the fungal nucleus. II. The kinetochore equivalent. (KCE). *J. Cell Sci.* **2,** 453–473.

Goode, B. L., Drubin, D. G., and Barnes, G. (2000). Functional cooperation between the microtubule and actin cytoskeletons. *Curr. Opin. Cell Biol.* **12,** 63–71.

Hagan, I., and Yanagida, M. (1990). Novel potential mitotic motor protein encoded by the fission yeast CUT7 positive gene. *Nature* **347,** 563–566.

Hagan, I. M., Riddle, P. N., and Hyams, J. S. (1990). Intramitotic controls in the fission yeast *Schizosaccharomyces pombe*: The effect of cell size on spindle length and the timing of mitotic events. *J. Cell Biol.* **110,** 1617–1621.

Harper, R. A. (1905). Sexual reproduction and the organization of the nucleus in certain mildews. Carnegie Institution of Washington Publication No. 37.

Heil-Chapdelaine, R. A., Adams, N. R., and Cooper, J. A. (1999). Formin' the connection between microtubules and the cell cortex. *J. Cell Biol.* **144,** 809–811.

Hildebrandt, E. R., and Hoyt, M. A. (2000). Mitotic motors in *Saccharomyces cerevisiae. Biochim. Biophys. Acta* **1496,** 99–116.

Howard, R. J., and Aist, J. R. (1979). Hyphal tip cell ultrastructure of the fungus *Fusarium:* Improved preservation by freeze-substitution. *J. Ultrastruct. Res.* **66,** 224–234.

Howard, R. J., and O'Donnell, K. L. (1987). Freeze substitution of fungi for cytological analysis. *Exp. Mycol.* **11,** 249–269.

Hoyt, M. A., and Geiser, J. R. (1996). Genetic analysis of the mitotic spindle. *Annu. Rev. Genet.* **30,** 7–33.

Inoué, S., and Ritter, H., Jr. (1975). Dynamics of mitotic spindle organization and function. *In* "Molecules and Cell Movement" (S. Inoué and R. E. Stephens, Eds.), pp. 3–30. Raven Press, New York.

Inoue, S., Turgeon, B. G., Yoder, O. C., and Aist, J. R. (1998a). Role of fungal dynein in hyphal growth, microtubule organization, spindle pole body motility and nuclear migration. *J. Cell Sci.* **111,** 1555–1566.

Inoue, S., Yoder, O. C., Turgeon, B. G., and Aist, J. R. (1998b). A cytoplasmic dynein required for mitotic aster formation in vivo. *J. Cell Sci.* **111,** 2607–2614.

Jensen, C. G., Aist, J. R., Bayles, C. H., Bollard, S. M., and Jensen, L. C. W. (1991). Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). III. Intermicrotubule bridges. *Protoplasma* **161,** 137–149.

Jin, Q. W., Fuchs, J., and Loidl, J. (2000). Centromere clustering is a major determinant of yeast interphase nuclear organization. *J. Cell Sci.* **113,** 1903–1912.

Kahana, J. A., Schnapp, B. J., and Silver, P. A. (1995). Kinetics of spindle pole body separation in budding yeast. *Proc. Natl. Acad. Sci. USA* **92,** 9707–9711.

Kirchner, J. S., Seiler, S., Fuchs, S., and Schliwa, M. (1999). Functional anatomy of the kinesin molecular in vivo. *EMBO J.* **18,** 4404–4413.

Li, Y.-Y., Yeh, E., Hays, T., and Bloom, K. (1993). Disruption of mitotic spindle orientation in a yeast dynein mutant. *Proc. Natl. Acad. Sci. USA* **90,** 10096–10100.

Maddox, P., Chin, E., Mallavarapu, A., Yeh, E., Salmpon, E. D., and Bloom, K. (1999). Microtubule dynamics from mating through the first zygotic division in the budding yeast *Saccharomyces cerevisiae. J. Cell Biol.* **144,** 977–987.

McIntosh, J. R., and Pfarr, C. M. (1991). Mitotic motors. *J. Cell Biol.* **115,** 577–586.

Meluh, P. B., and Rose, M. D. (1990). *KAR3,* a kinesin-related gene required for yeast nuclear fusion. *Cell* **60,** 1029–1041.

Nabeshima, K., Nakagawa, T., Straight, A. F., Murray, A., Chikashige, Y., Yamashita, Y. M., Hiraoka, Y., and Yanagida, M. (1998). Dynamics of centromeres during metaphase–anaphase transition in fission yeast: Dis1 is implicated in force balance in metaphase bipolar spindle. *Mol. Biol. Cell* **9,** 3211–3225.

O'Connell, M. J., Meluh, P. B., Rose, M. D., and Morris, N. R. (1993). Suppression of the *bim*C4 mitotic spindle defect by deletion of klpA, a gene encoding a KAR3-related kinesin-like protein in *Aspergillus nidulans. J. Cell Biol.* **120,** 153–162.

O'Donnell, K. (1994). A reevaluation of the mitotic spindle pole body cycle in *Tilletia caries* based on freeze-substitution techniques. *Can. J. Bot.* **72,** 1412–1423.

O'Toole, E. T., Winey, M., and McIntosh, J. R. (1999). High-voltage electron tomography of spindle pole bodies and early mitotic spindles in the yeast *Saccharomyces cerevisiae. Mol. Biol. Cell* **10,** 2017–2031.

Plamann, M., Minke, P. F., Tinsley, J. H., and Bruno, K. S. (1994). Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. *J. Cell Biol.* **127,** 139–149.

Rebhun, K. I. (1972). Polarized intracellular particle transport: Saltatory movements and cytoplasmic streaming. *Int. Rev. Cytol.* **32,** 93–137.

Sandrock, T. M., Yoder, O. C., Turgeon, B. G., and Aist, J. R. (1999). Subtle mitotic phenotypes of a Kar3/KlpA-like mutant of *Nectria haematococca. Mol. Biol. Cell* **10,** 128a.

Saunders, W. S. (1993). Mitotic spindle pole separation. *Trends Cell Biol.* **3,** 432–437.

Saunders, W. S., Koshland, D., Eshel, D., Gibbons, I. R., and Hoyt, M. A. (1995). *Saccharomyces cerevisiae* kinesin- and dynein-related proteins required for anaphase chromosome segregation. *J. Cell Biol.* **128,** 617–624.

Sharp, D. J., Rogers, G. C., and Scholey, J. M. (2000). Microtubule motors in mitosis. *Nature* **407,** 41–47.

Shaw, S. L., Yeh, E., Maddox, P., Salmon, E. D., and Bloom, K. (1997). Astral microtubule dynamics in yeast: A microtubule-based searching mechanism for spindle orientation and nuclear migration into the bud. *J. Cell Biol.* **139,** 985–994.

Straight, A. F., Maarshall, W. F., Sedat, J. W., and Murray, A. W. (1997). Mitosis in living budding yeast: Anaphase A but no metaphase plate. *Science* **277,** 574–578.

Su, S. S. Y., and Yanagida, M. (1997). Mitosis and cytokinesis in the fission yeast, *Schizosaccharomyces pombe*. *In* "The Molecular and Cellular Biology of the Yeast *Saccharomyces,*" Cold Spring Harbor Monograph Series No. 21, pp. 765–825. Cold Spring Harbor Laboratory Press, Plainview, NY.

Wilson, C. L., and Aist, J. R. (1967). Motility of fungal nuclei. *Phytopathology* **57,** 769–771.

Winey, M., and O'Toole, E. T. (2001). The spindle cycle in budding yeast. *Nat. Cell Biol.* **3,** E23–E27.

Winey, M., Mamay, C. L., O'Toole, E. T., Mastronarde, D. N., Giddings, T. H. J., McDonald, K. L., and McIntosh, J. R. (1995). Three-dimensional ultrastructural analysis of the *Saccharomyces cerevisiae* mitotic spindle. *J. Cell Biol.* **129,** 1601–1615.

Wu, Q., Sandrock, T. M., Turgeon, B. G., Yoder, O. C., Wirsel, S. G., and Aist, J. R. (1998). A fungal kinesin required for organelle motility, hyphal growth, and morphogenesis. *Mol. Biol. Cell* **9,** 89–101.

Xiang, X., Beckwith, S. M., and Morris, N. R. (1994). Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulans. Proc. Natl. Acad. Sci. USA* **91,** 2100–2104.

Xiang, X., Han, G., Winkelmann, D. A., Zuo, W., and Morris, N. R. (2000). Dynamics of cytoplasmic dynein in living cells and the effect of a mutation in the dynactin complex actin-related protein Arp1. *Curr. Biol.* **10,** 603–606.

Yeh, E., Skibbens, R. V., Cheng, J. W., Salmon, E. D., and Bloom, K. (1995). Spindle dynamics and cell cycle regulation of dynein in the budding yeast, *Saccharomyces cerevisiae. J. Cell Biol.* **130,** 687–700.

Yeh, E., Yang, C., Chin, E., Maddox, P., Salmon, E. D., Lew, D. J., and Bloom, K. (2000). Dynamic positioning of mitotic spindles in yeast: Role of microtubule motors and cortical determinants. *Mol. Biol. Cell* **11,** 3949–3961.

This Page Intentionally Left Blank

INDEX

D

E

F

G

H

J

K

L

M

N

O

P

R

S

T

V