Small GTP-Binding Proteins

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Small GTP-binding proteins (G proteins) exist in eukaryotes from yeast to human and constitute a superfamily consisting of more than 100 members. This superfamily is structurally classified into at least five families: the Ras, Rho, Rab, Sar1/Arf, and Ran families. They regulate a wide variety of cell functions as biological timers (biotimers) that initiate and terminate specific cell functions and determine the periods of time for the continuation of the specific cell functions. They furthermore play key roles in not only temporal but also spatial determination of specific cell functions. The Ras family regulates gene expression, the Rho family regulates cytoskeletal reorganization and gene expression, the Rab and Sar1/Arf families regulate vesicle trafficking, and the Ran family regulates nucleocytoplasmic transport and microtubule organization. Many upstream regulators and downstream effectors of small G proteins have been isolated, and their modes of activation and action have gradually been elucidated. Cascades and cross-talks of small G proteins have also been clarified. In this review, functions of small G proteins and their modes of activation and action are described.

I. INTRODUCTION

Small GTP-binding proteins (G proteins) are monomeric G proteins with molecular masses of 20–40 kDa. The Ha-Ras and Ki-Ras genes were first discovered as the v-Ha-Ras and v-Ki-Ras oncogenes of sarcoma viruses around 1980 (111, 660). Their cellular oncogenes were then identified in humans, and their mutations were furthermore found in some human carcinomas (146, 252, 499, 561, 626, 661). The mutated forms were subsequently shown to stimulate proliferation and transformation of cultured cells (71, 84, 182, 681). Moreover, the mutated forms were shown to induce cell differentiation in neuronal cells (41, 249, 523). These findings drew the attention of many scientists not only in the cancer research field but also in many other fields. Finally, these Ras proteins were shown to be related to the heterotrimERIC G proteins, such as Gs and Gi, and G proteins involved in protein synthesis, such as elongation factor Tu (EF-Tu) (222, 644, 659).

The Rho gene was discovered as a homolog of the Ras gene in Aplysia in 1985 (421); the YPT1 gene, which had been discovered as an open reading frame between the actin and tubulin genes in the yeast Saccharomyces cerevisiae (S. cerevisiae) in 1983 (208), was identified to encode a small G protein in 1986 (641); Arf protein, which was purified as a cofactor for the cholera toxin-catalyzed ADP-ribosylation of Gs in 1984 (326), was identified to encode a small G protein in 1986 (327). The SEC4 gene, which had been isolated as a gene involved in secretion in the yeast in 1980 (527), was identified to encode a small G protein in 1987 (623). These results suggested the presence of a big family of Ras-like small G proteins. Actually, many small G proteins were systematically isolated by molecular biological (100, 101, 105, 551, 573) and biochemical (291, 337, 344, 534, 535, 793, 797) methods.

Now, more than 100 small G proteins have been identified in eukaryotes from yeast to human, and they comprise a superfamily (60, 250, 701). The members of this superfamily are structurally classified into at least five families: the Ras, Rho, Rab, Sar1/Arf, and Ran families (Table 1 and Fig. 1). In the yeast S. cerevisiae, sequence analysis against complete genomic sequence has revealed that there are 4 Ras family members, 6 Rho family members, 11 Rab family members, 7 Sar1/Arf family members, and 2 Ran family members (210, 383). The functions of many small G proteins have recently been elucidated: the Ras subfamily members (Ras proteins) of the Ras family mainly regulate gene expression, the Rho/Rac/Cdc42 subfamily members (Rho/Rac/Cdc42 proteins) of the Rho family regulate both cytoskeletal reorganization and gene expression, the Rab and Sar1/Arf family members (Rab and Sar1/Arf proteins) regulate intracellular vesicle trafficking, and the Ran family members (Ran) regulate nucleocytoplasmic transport during the G1, S, and G2 phases of the cell cycle and microtubule organization during the M phase. Many upstream regulators and downstream effectors of small G proteins have been identified, and modes of activation and actions have gradually been elucidated. In this review, functions of small G proteins and their modes of activation and action are described. However, this review may not cover all detailed information regarding each small G protein; readers may refer to other recent excellent reviews (3, 49, 58, 80, 82, 139, 325, 417, 420, 428, 483, 491, 519, 536, 630, 713, 744, 758).

As to nomenclature, the term small GTPases is often used, but “small G proteins” is used here because small G proteins are referred to as G proteins in many papers.
proteins have both GDP/GTP-binding and GTPase activities. In many cases, the GTPase activity is necessary for the termination of the functions of small G proteins, but not essential for them to perform their functions. From this point of view, the term GTPase is misleading. "G proteins" represent heterotrimeric G proteins and "small GTP-binding proteins" should be used, but just for simplicity "small G proteins" is used here. G proteins used here include heterotrimeric G proteins (223), G proteins involved in protein synthesis (338), and small G proteins. Guanine nucleotide exchange factor (GEF) is often used, but guanine nucleotide exchange protein (GEP) is used here, because all GEFs thus far found are proteins and "GEPs" is a more correct term.

II. GENERAL PROPERTIES

A. Structure

A comparison of the amino acid sequences of Ras proteins from various species has revealed that they are conserved in primary structures and are 30–55% homologous to each other. Among Ras proteins, each protein shares relatively high (50–55%) amino acid identity, whereas Rab and Rho/Rac/Cdc42 proteins share ~30% amino acid identity with Ras proteins (250, 742). Nevertheless, like other G proteins, all small G proteins have consensus amino acid sequences responsible for specific interaction with GDP and GTP and for GTPase activity, which hydrolyzes bound GTP to GDP and P_i (61, 701, 742) (Fig. 2A). Moreover, they have a region interacting with downstream effectors. In addition, small G proteins belonging to Ras, Rho/Rac/Cdc42, and Rab proteins have sequences at their COOH termini that undergo posttranslational modifications with lipid, such as farnesyl, geranylglycerol, palmitoyl, and methyl moieties, and proteolysis (89, 228, 427, 701, 811) (Fig. 3). Arf proteins have an NH_2-terminal Gly residue that is modified with myristic acid (493). Sar1 and Ran do not have such sequences to direct posttranslational modifications.

TABLE 1. The small G protein superfamily

<table>
<thead>
<tr>
<th>Ras Family</th>
<th>Rho Family</th>
<th>Rab Family</th>
<th>Sar/Arf Family</th>
<th>Ran Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-Ras</td>
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<td>RhoA</td>
<td>Rab1A</td>
<td>Rab11A</td>
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<td>RhoB</td>
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<td>Rab11B</td>
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<td>N-Ras</td>
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<td>RhoC</td>
<td>Rab2</td>
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<td>RhoD</td>
<td>Rab3A</td>
<td>Rab13</td>
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<tr>
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<td>Rab3B</td>
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<td>Rab8</td>
<td>Rab23</td>
<td>Arf6</td>
</tr>
<tr>
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<td>Rnd1/</td>
<td>Rab9</td>
<td>Rab24</td>
<td>Arf7</td>
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See References 251a and 810a.
bond with the γ-phosphate of GTP. Binding of c-Raf-1, an effector of Ras proteins (see below), stabilizes the effector loop in the active conformation (459).

The COOH-terminal regions are classified into at least four groups: 1) Cys-A-A-X (A, aliphatic acid; X, any amino acid); 2) Cys-A-Leu/Phe; 3) Cys-X-Cys; and 4) Cys-Cys (89, 228, 427, 701) (Fig. 3). The Cys-A-A-X structure is furthermore subclassified into two groups: one has an additional Cys residue upstream of the Cys residue of the Cys-A-A-X structure (1a), and the other has a polybasic region (1b). In the case of the Cys-A-A-X structure, Ha-Ras and Ki-Ras are first farnesylated at the Cys residue followed by the proteolytic removal of the A-A-X portion and the carboxymethylation of the exposed Cys residue.

B. A Role as Molecular Switches

According to the structures of small G proteins, they have two interconvertible forms: GDP-bound inactive and GTP-bound active forms (60, 250, 701) (Fig. 4). An upstream signal stimulates the dissociation of GDP from the GDP-bound form, which is followed by the binding of GTP, eventually leading to the conformational change of the downstream effector-binding region so that this region interacts with the downstream effector(s). This interaction causes the change of the functions of the downstream effector(s). The GTP-bound form is converted by the action of the intrinsic GTPase activity to the GDP-bound form, which then releases the bound downstream effector(s). In this way, one cycle of activation and inactivation is achieved, and small G proteins serve as molecular switches that transduce an upstream signal to a downstream effector(s).

Thus the rate-limiting step of the GDP/GTP exchange reaction is the dissociation of GDP from the GDP-bound form. This reaction is extremely slow and therefore stimulated by a regulator, named GEP (also called GEF or guanine nucleotide releasing factor), of which activity is often regulated by an upstream signal. GEP first interacts with the GDP-bound form and releases bound GDP to form a binary complex of a small G protein and GEP. Then, GEP in this complex is replaced by GTP to form the active complex of a small G protein and GEP.

(90, 201, 247, 256, 313). Ha-Ras has an additional Cys residue that is further palmitoylated (256). The Cys-A-A-Leu structure of Rap1 is first geranylgeranylated followed by the same modifications (336). Both Cys residues of the Cys-X-Cys structure of Rab3A are geranylgeranylated, and the COOH-terminal Cys residue is carboxymethylated (177). Both Cys residues of the Cys-Cys structure of Rab1 are geranylgeranylated, but the COOH-terminal Cys residue is not carboxymethylated (672). The lipid modifications of these small G proteins are necessary for their binding to membranes and regulators and for their activation of downstream effectors as described below (89, 228, 257, 427, 701, 702, 811).

The farnesyl moiety is derived from farnesyl pyrophosphate, an intermediate product of the mevalonate pathway which produces cholesterol from mevalonate (231). Mevalonate is produced from 3-hydroxy-3-methylglutaryl-CoA by the action of 3-hydroxy-3-methylglutaryl-coenzyme reductase. A specific inhibitor for this enzyme, named pravachol, is used as a very effective drug for arteriosclerosis (171, 231). The geranylgeranyl moiety is derived from geranylgeranyl pyrophosphate, which is an intermediate product of the synthesis of dolichol and ubiquinone (220, 231). The palmitoyl moiety is derived from palmitoyl CoA. The methyl moiety is derived from S-adenosyl-methionine. The enzymes that transfer the prenyl moieties have been isolated and characterized (811). The farnesylation of the Cys-A-A-X structure is catalyzed by farnesyltransferase, the geranylgeranylation of the Cys-A-A-Leu structure is catalyzed by geranylgeranyltransferase I, and the prenylation of the Cys-X-Cys and Cys-Cys structures is catalyzed by geranylgeranyltransferase II. Farnesyltransferase and geranylgeranyltransferase I consist of two subunits, α and β subunits, and the α-subunits of both enzymes are identical (648). Geranylgeranyltransferase II consists of three subunits, originally termed component A but recently renamed Rab escort protein I (Rep1), and α- and β-subunits (289, 645, 646, 672). Rep1 binds unprenylated Rab proteins, presents them to the catalytic αβ-subunits, and remains bound to Rab proteins after the geranylgeranyl transfer reaction (20). In cells, Rab GDP dissociation inhibitor (GDI) (see below) may dissociate this product from Rep1, allowing multiple cycles of catalysis. The human Rep1 gene has been identified by positional cloning as that responsible for choroideremia, which is an X-linked form of retinal degeneration (131, 132, 187). Loss of Rep1 activity causes the reduced prenylation of Ram/Rab27, which is expressed at high levels in the retinal cell layers, and the degeneration of this protein in the progression of this disease (647). Palmitoyltransferase that palmitoylates Ha-Ras has been purified (406), but it is not yet known whether this enzyme is the one that functions in vivo. Methyltransferases that transfer the methyl moieties to small G proteins having Cys-A-A-X, Cys-A-A-Leu, and Cys-X-Cys structures have not been well characterized. A protease, Rce1, that removes the A-A-X and A-A-Leu portions of Ha-Ras, N-Ras, Ki-Ras, and Rap1B, has recently been identified (345, 557).

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member or subfamily of small G proteins (56, 75, 762), but some GEPs, such as Dbl, a GEP active on Rho/Rac/Cdc42 proteins, show wider substrate specificity (259, 788). The GDP/GTP exchange reactions of Rho/Rac/Cdc42 and Rab proteins are furthermore regulated by another type of regulator, named Rho GDI and Rab GDI, respectively (28, 206, 453, 629, 732). This type of regulator inhibits both the basal and GEP-stimulated dissociation of GDP from the GDP-bound form and keeps the small G protein in the GDP-bound form. Rho GDI and Rab GDI show wider substrate specificity than GEPs and GTPase-activating proteins (GAPs) and are active on all Rho/Rac/Cdc42 and Rab proteins, respectively (18, 275, 391, 627, 629, 732, 737). Thus the activation of Rho/Rac/Cdc42 and Rab proteins is regulated by positive and negative regulators.

Recently, Ran GDI, p10/NTF2, has also been reported (125, 485, 563, 789), but GDIs have not been identified for other small G proteins. The GTPase activity of each small G protein is variable but relatively very slow and is stimulated by GAPs. Most GAPs, such as Ras GAP and Rab3 GAP, are specific for each member or subfamily of small G proteins (56, 205, 727), but some GAPs, such as p190, a GAP active on Rho/Rac/Cdc42 proteins, show wider substrate specificity (656).

C. Localization

Small G proteins as well as heterotrimeric G proteins are present only in eukaryotes from yeast to human, although G proteins involved in protein synthesis such as elongation factors exist in both prokaryotes and eukaryotes. Most small G proteins are widely distributed in mammalian cells, and most cells have the Ras, Rho, Rab, Sar1/Arf, and Ran families, although expression levels of their members may vary from one type to another. A few members show tissue-specific expression; for instance, Rab3A is expressed in cells having a regulated secretion pathway, such as neurons, neuroendocrine cells, and exocrine cells (140, 183, 476, 477, 625). Rab17 is detected in epithelial cells (413). Most small G proteins are localized either in the cytosol or on membranes. Ran is localized either in the cytosol or in the nucleus. Each small G protein is localized to a specific membrane. Ras proteins are localized at the cytoplasmic face of the plasma membrane. This localization is mediated by the posttranslational modifications with lipid. The farnesyl moiety of Ha-Ras and Ki-Ras alone is not sufficient for their binding to the membrane (256). In the case of Ha-Ras, both the farnesyl and palmitoyl moieties are necessary, whereas in the case of Ki-Ras, both the farnesyl moiety and the neighboring clustered polybasic amino acids are necessary. The farnesyl and palmitoyl moieties may interact with the acyl moieties of the phospholipids, whereas the polybasic amino acids may interact with the polar head groups of the acidic phospholipids. The methyl moiety also contributes markedly to efficient membrane association (255). Rap1 is geranylgeranylated and has clustered polybasic amino acids. Most Rab proteins have either a Cys-X-Cys or Cys-Cys structure of which Cys residues are both geranylgeranylated. These small G proteins are localized at the cytoplasmic faces of distinct membrane compartments. It has not been experimentally clarified how Rap1 and Rab proteins exactly interact with the membranes, but it is likely that both the prenyl moiety and the polybasic region or two prenyl moieties are necessary. In contrast, Arf proteins have one myristoyl moiety and Sar1 has no lipid moiety, but they interact with the cytoplasmic faces of membranes. Arf proteins interact with membrane lipids by its myristoylated and amphipathic NH2-terminal helix (21, 47). In the case of Sar1, it may interact with the phospholipid through only peptide region. Small G proteins, such as Rho/Rac/Cdc42 and Rab proteins, located on the plasma membrane and the cytosol are translocated between these two sites. Ran is also translocated between the cytosol and the nucleus through the nuclear pore complexes (NPCs).

III. RAS PROTEINS AS REGULATORS OF GENE EXPRESSION

A. Outline

Three Ras proteins are now known, Ha-Ras, Ki-Ras, and N-Ras, which are capable of transforming mammalian cells when activated by point mutations (71, 84, 182, 681). In the yeast S. cerevisiae, there are two members of Ras proteins, Ras1 and Ras2, that are essential for cell viability, and these yeast genes are functionally replaceable by mammalian genes (141, 577). The downstream effector of Ras proteins was first identified to be adenylate cyclase in the yeast S. cerevisiae (66, 721). Mammalian adenylate...
cyclase is directly regulated by heterotrimeric G proteins, but not by Ras proteins. Subsequently, genetic, cell biological, and biochemical studies in Caenorhabditis elegans, Drosophila, and mammalian cells established the mode of action of Ras proteins; they directly bind to and activate Raf protein kinase (82, 151a, 254a, 743, 759, 767, 815), which then induces gene expression through the mitogen-activated protein (MAP) kinase cascade in response to various extracellular signaling molecules (145, 299, 416). Other studies have clarified that Ras proteins regulate not only cell proliferation but also differentiation (41, 249, 523), morphology (41a, 182, 779), and apoptosis (334). Ras proteins regulate these functions mainly through gene expression, but it has not been established whether the Ras protein-mediated morphological changes are a direct effect or an indirect effect through gene expression. Another characteristic feature of Ras proteins is that the mutations of their genes and their regulator genes cause human cancers (9, 42, 59, 445, 607, 756, 786). Thus Ras proteins are crucially important molecules not only for biology but also for human health.

B. Ras Protein Cycle: Activation/Inactivation

Ras protein activity is regulated by GEPs and GAPs, and activation is induced by a large variety of extracellular signals, most notably signals that activate receptors with intrinsic or associated tyrosine kinase activity (168, 207, 399, 549, 617) (Fig. 5). Phosphotyrosines serve as docking sites for the adaptor proteins, such as GRB2 and SHC/GRB2 complex, which then recruit SOS, the most characterized Ras GEP, from the cytosol to produce a receptor-adaptor-GEP complex. SOS recruited to the plasma membrane then stimulates a Ras protein located at the cytoplasmic face of the plasma membrane and converts it from the GDP-bound form to the GTP-bound form. It is believed that GRB2 recruits SOS from the cytosol to the plasma membrane without affecting its GEP activity, but the possibility has not been totally excluded that GRB2 both recruits and activates SOS. Receptors not directly associated with tyrosine kinases, such as T-cell receptors, may activate Ras proteins indirectly through Src-like tyrosine kinases or ligand-independent activation of receptor tyrosine kinases (243, 690, 774). Moreover, heterotrimeric G protein-coupled receptors, such as α-adrenergic receptors, muscarinic acetylcholine receptors, and lysophosphatidic acid, have also been shown to activate Ras proteins (266, 285, 294). In addition, an increase of cytoplasmic Ca\textsuperscript{2+} induced by activation of these receptors in neurons also induces activation of another type of GEPs, p140 Ras GRF, that contains an IQ motif regulated by calcium-bound calmodulin (178).

After the GTP-bound forms of Ras proteins accomplish their effects on downstream effector(s), they are converted to the GDP-bound form by the action of Ras GAPs. However, how termination of Ras protein signaling is achieved is not fully understood. Phosphorylation of SOS by the Raf/MAP kinase pathway (see below) may induce the dissociation of SOS from GRB2 (281, 356), and another possible mechanism is that Ras GAPs are activated by their binding to tyrosine-phosphorylated growth factor receptors such as the platelet-derived growth factor (PDGF) receptor (17, 332).

Three GEPs of Ras (SOS, Cdc25, and Ras GRF) have

\[ \text{Extracellular signal} \]

\[ \text{Receptor} \]

\[ \text{SHC, GRB2, SOS} \]

\[ \text{Ras} \]

\[ \text{GDP} \]

\[ \text{GTP} \]

\[ \text{GAP} \]

\[ \text{GDP} \]

\[ \text{GTP} \]

\[ \text{Raf} \]

\[ \text{MEK} \]

\[ \text{MAP kinase} \]

\[ \text{Gene expression} \]

\[ \text{One directional regulation} \]

\[ \text{Modifiers} \]

\[ 14-3-3 \]

\[ \text{KSR (Drosophila)} \]

\[ \text{CNK/MAGUIN} \]

\[ \text{Membrane} \]

\[ \text{PI} \]

\[ \text{GTP} \]

\[ \text{GDP} \]

\[ \text{Raf} \]

\[ \text{MEK} \]

\[ \text{MAP kinase} \]

\[ \text{Gene expression} \]

\[ \text{One directional regulation} \]

\[ \text{FIG. 5. Mode of action of Ras proteins in gene expression. Ras, Ras proteins.} \]
been discovered to date. The first GEP for Ras, Cdc25, has been identified genetically in S. cerevisiae (67, 81, 605). Cdc25 has a GEP domain that is required for its catalytic activity, located in its COOH-terminal region. In addition, Cdc25 has an SH3 domain in the NH2-terminal region. In higher eukaryotes, in addition to mammalian Cdc25 (mCdc25), two different types of proteins with homology to Cdc25 have been found. The first group includes SOS. SOS was first identified in Drosophila. Genetic studies have shown that SOS is downstream of Sevenless, a receptor tyrosine kinase, which is homologous to the epidermal growth factor (EGF) receptor (609, 669). One human and two murine homologs of SOS have been cloned. SOS has one pleckstrin homology (PH) domain that may interact with a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), to determine its activity or localization of p120 Ras GAP has been demonstrated upon tyrosine phosphorylation. Protein tyrosine kinases may control the activity of p120 Ras GAP by forming a complex with it. In fact, p120 Ras GAP forms a complex with activated PDGF receptors (332, 339). Two other proteins have been found to associate with p120 Ras GAP. One is p62 Dok, a docking protein that contains multiple tyrosine phosphorylation sites for its binding to the SH2 domains of p120 Ras GAP and Nck (87, 796). The other is p190 Rho GAP; however, the precise role of the interaction between p190 Rho GAP and p120 Ras GAP is not fully understood (486, 656). p120 Ras GAP-deficient mice die during embryonic development, and the ability of endothelial cells to organize a vascular network is severely impaired in addition to extensive neuronal cell death in these animals (268). Another Ras GAP named GAP1m, which shows a high degree of similarity to the Drosophila Gap1 gene, has been also identified (426). In addition to the GAP catalytic domain, GAP1m has two domains with sequences closely related to those of the phospholipid-binding domain of synaptotagmin and a region with similarity to the unique domain of Btk tyrosine kinase.

S. cerevisiae contains two Ras GAPS, Ira1 and Ira2 (711, 712), which contain domains homologous to the COOH terminus of p120 Ras GAP. In wild-type cells, Ras proteins are generally inactive, but in the absence of either the Ira gene product, they accumulate in their GDP-bound state, becoming hyperactive and leading to overproduction of cAMP. In yeast, at least, Ras GAPS are therefore not the effectors of Ras proteins; rather, they serve as negative regulators.

Neurofibromin (NF1), the human protein defective in von Recklinghausen neurofibromatosis (a benign tumor), contains a domain homologous to the catalytic domains of p120 Ras GAP, Ira1, and Ira2 (445, 786). Like p120 Ras GAP itself, neurofibromin possesses GAP activity in vitro and can complement the loss of Ira function in the yeast S. cerevisiae, indicating that it may be the mammalian homolog of Ira1 and Ira2 (34). These proteins share regions of significant similarity outside the GAP-related domain and presumably perform similar functions.

Small G protein GDP dissociation stimulator (Smg GDS) is a regulator that is entirely distinct from GEPs, GDIs, and GAPS (328, 795). Smg GDS has two biochemical activities on a group of small G proteins including Ki-Ras and the Rho/Rac/Cdc42/Rap1 proteins: one is to stimulate their GDP/GTP exchange reactions and the other is to inhibit their binding to membranes (18, 275, 479, 480, 788). A detailed kinetic study of Smg GDS with Ki-Ras as a substrate has revealed that it interacts with not only GDP-Ki-Ras and the guanine nucleotide-free Ki-Ras but also GTP-Ki-Ras, under the conditions where a Ras GEP, mCdc25, does not form a ternary complex with GTP-Ki-Ras (506). This property of Smg GDS suggests that it stimulates the GDP/GTP exchange reaction only once. The physiological role of Smg GDS remains to be established, although it shows mitogenic and transforming activities in cooperation with Ki-Ras in fibroblasts (198). Studies on Smg GDS-deficient mice have revealed that mice die of heart failure shortly after birth (708). En-
hanced apoptosis is observed in at least heart, thymus, and neuron in Smg GDS-deficient mice. This phenotype is apparently similar to those of Ki-Ras-deficient mice (321, 362), providing another line of evidence that Smg GDS plays a role in Ki-Ras-mediated signaling pathway in vivo.

C. Raf Protein Kinase Activation by Ras Proteins

Ras proteins mediate their effects on cell proliferation mainly by activation of a cascade of protein kinases: Raf protein kinase (c-Raf-1, A-Raf, and B-Raf), MEK (MAP kinase kinases 1 and 2), and MAP kinase. Ras proteins activate this protein kinase cascade by directly binding to Raf proteins (743, 759, 767, 815). Raf proteins then phosphorylate and activate MEK (145, 299, 378), which then phosphorylates and activates MAP kinase (134, 450). The activated MAP kinase translocates to the nucleus, where it phosphorylates and stimulates the activity of various transcription factors, including Elk-1 (442). The recent observation that Ras proteins interact with two distinct NH2-terminal regions of Raf-1 suggests that Ras proteins promote more than just membrane translocation of Raf-1 and instead may also facilitate the subsequent events that lead to Raf-1 activation (495, 513, 759).

The initial step of the Raf-1/MEK/MAP kinase cascade is the activation of Raf-1 by direct interaction with a GTP-Ras protein (GTP-Ras) (743, 759, 767, 815). The interaction with GTP-Ras localizes Raf-1 to the plasma membrane (389, 687). The first described and best-characterized Ras-binding domain (RBD) is contained within residues 51–131 of Raf-1 (121, 513, 759). The association of the RBD with the Ras effector domain is a high-affinity interaction that is mediated primarily by residues Gln-66, Lys-84, and Arg-89 of Raf-1 (55). The interaction between the RBD and GTP-Ras appears to then allow for a second RBD of Raf-1 to contact GTP-Ras (72, 159). This second RBD (residues 139–184 of Raf-1) encompasses the conserved Cys finger motif within the Raf-1 NH2 terminus and is referred to as the Cys-rich domain (CRD) (495). In terms of the Ras-Raf-1 interaction, the CRD associates with different residues of GTP-Ras than does the RBD (159), and posttranslational modifications of Ras proteins may be important for CRD binding (297, 376). Thus, in the full-length molecule, the CRD is inaccessible for GTP-Ras binding, but either mutational events or RBD binding can unmask the CRD and allow it to interact with GTP-Ras. Thus, for the Ras-Raf-1 interaction to result in Raf-1 activation, binding to both the RBD and the CRD appears to be required.

Ha-Ras is localized at the cytoplasmic surface of the plasma membrane, while mutant forms of Ha-Ras, which lack posttranslational lipid modification, are cytosolic and lack biological activity. These findings suggest that posttranslational lipid modifications of Ha-Ras are required for both its localization and biological activity. Furthermore, lipid-modified Ras proteins have been shown to more efficiently activate adenylate cyclase in the yeast system (288, 376). Posttranslational modifications of Ki-Ras are also required for MAP kinase activation in a cell-free system (309). In addition, posttranslational modifications of Ha-Ras are required for activation of, but not for association with, Raf protein kinase (392, 547).

The intracellular signals that couple growth factors to MAP kinase may determine the different effects of growth factors; for example, transient activation of MAP kinase by EGF stimulates proliferation of PC12 cells, whereas sustained activation of MAP kinase by nerve growth factor (NGF) induces differentiation of PC12 cells. Activation of MAP kinase by NGF involves two distinct pathways: the initial activation of MAP kinase requires Ras proteins, but its activation is sustained by Rap1 (806) (see below). Rap1 is activated by C3G, a GEP for Rap1, and forms a stable complex with B-Raf (806). Activation of B-Raf by Rap1 represents a common mechanism to induce sustained activation of the MAP kinase cascade.

D. Modifiers of the Ras Protein-Induced Raf Protein Kinase Activation

In addition to Ras proteins, a protein named 14–3-3 seems to also interact with Raf-1 and activate it (176, 197). 14–3-3 is a specific phosphoserine-binding protein (500). Raf-1 itself contains two phosphorylation sites that may interact with 14–3-3. 14–3-3 may have two different roles: first, 14–3-3 may be required for maintaining Raf-1 in an inactive conformation, as Raf-1 that is unable to stably interact with 14–3-3 is activated (467). In response to signaling events and Raf protein activation, 14–3-3 may subsequently play a second role in facilitating activation of Raf-1 and stabilizing activated Raf-1.

The observation that Raf-1 becomes hyperphosphorylated in response to many signaling events (492) has long suggested that phosphorylation plays a role in regulating Raf-1 activity. Mechanisms by which phosphorylation could regulate Raf1 function include direct alteration of the intrinsic activity of Raf-1 and alteration of critical protein interactions, such as with 14–3-3. The rapid and transient nature of Raf-1 activation further complicates the issue, making it difficult to distinguish between activating and inactivating modifications. Nevertheless, by the use of overexpression systems and mutational analysis, the phosphorylation of tyrosine residues 340 and 341 has been shown to enhance the catalytic activity of Raf-1 (441). The tyrosine kinases implicated in phosphorylating Raf-1, and thereby enhancing its activity, include members of the Src kinase family (441, 562, 576).

A novel protein kinase that functions downstream of
Ras proteins, kinase suppressor of Ras (KSR), has recently been identified by genetic screening as a suppressor of phenotypes caused by an activated Ras protein in both Drosophila and C. elegans (366, 694, 717). Epistasis analysis in Drosophila suggests that KSR functions downstream of Drosophila Ras-I but upstream or in parallel to Raf protein (717). Characterization of a mouse KSR homolog suggests that KSR facilitates signal transmission between Raf proteins, MEK, and MAP kinase (718). In addition, upon Ras protein activation, KSR translocates to the plasma membrane, where it forms a stable complex with Raf proteins (718). Moreover, KSR, via its kinase domain, forms a stable complex with MEK in the cytosol of quiescent cells (144). Therefore, in response to an activated Ras protein, KSR might shuttle MEK from the cytosol to activated Raf proteins at the membrane.

With the use of a screen for eye development defects in Drosophila, the connector enhancer of KSR (CNK) protein has been identified as an enhancer of a KSR dominant negative mutant phenotype (719). Mutation of CNK suppresses the phenotype of activated Ras proteins or Sevenless but not Raf proteins, suggesting that it acts upstream of Raf proteins. CNK has several protein interaction domains. These domains include a sterile alpha motif (SAM) domain, a PSD-95/Dlg-A/ZO-1 (PDZ) domain, two proline-rich (potential SH3-binding) domains, and a PH domain; such domains are found in many proteins involved in signaling and suggest further interactions of CNK with other proteins and small molecules. In two-hybrid assays in S. cerevisiae, a COOH-terminal portion of CNK that contains the PH domain interacts with the Raf kinase domain (719). Thus the SAM, PDZ, and novel domains might be available for other interactions, although it is not known whether CNK also binds other proteins in the Ras-Raf signaling pathway. CNK has a molecular structure similar to that of recently identified rat neuronal proteins, named MAGUINs, that interact with the PDZ domains of PSD-95/SAP90 and S-SCAM (802). MAGUINs interact with c-Raf-1 but do not affect its enzymatic activity (803). PSD-95/SAP90 and S-SCAM are neuronal membrane-associated guanylate kinases, and these proteins function as synaptic scaffolding proteins (264). In fact, PSD-95/SAP90 further interacts with synGAP, which regulates the activity of Ras proteins (346). Therefore, MAGUINs may also bind Raf proteins and link it to PSD-95/SAP90 and S-SCAM in synaptic junctions.

E. Other Effectors of Ras Proteins

A variety of candidate Ras protein effectors have been reported in addition to Raf proteins. These include Raf GDS (279, 342, 676), RIN1 (254), and phosphatidylinositol (PI) 3-kinase (608). AF6/Canoe is also suggested to be a binding partner of Ras proteins (373, 746), but this result has been called into question (434). It has recently been shown that Rap1 shows a much higher affinity to AF6 than Ras proteins do (404). p120 Ras GAP may participate in Ras protein-mediated gene expression, although it is still unclear whether p120 Ras GAP is a regulator, an effector, or both for Ras proteins. In contrast, activation of PI 3-kinase by Ras proteins may promote cell survival (334, 608). However, it has not been established whether these effector molecules other than Raf proteins really play a role in the downstream pathway of Ras proteins.

F. Transport of Newly Synthesized Ras Proteins
From the Endoplasmic Reticulum to the Plasma Membrane

The plasma membrane localization of Ras proteins is crucial for their functions. The mechanism by which Ras proteins get to the plasma membrane has not fully been understood. It has recently been shown that Ras proteins do not directly travel to the plasma membrane from the cytosol, but interact with intracellular membranes (25, 114). Ras proteins first associate with the endoplasmic reticulum and then with the Golgi apparatus. The initial association of Ras proteins with the endoplasmic reticulum requires only the COOH-terminal Cys-A-A-X structure and farnesylation. N-Ras and Ha-Ras seem to be transported by exocytic vesicles following association with the endoplasmic reticulum and the Golgi apparatus. Ki-Ras takes a faster route that may not involve the Golgi apparatus. The hypervariable domains of the three Ras proteins are necessary and sufficient to account for their differential localizations. Inhibition of vesicle transport with brefeldin A (BFA), an inhibitor of Arf protein GEP (see below), blocks the transit of N-Ras to the plasma membrane, demonstrating the importance of vesicle transport for N-Ras function. Carboxymethylation and A-A-X proteolysis are also necessary for proper association with the plasma membrane (255).

G. Ras Proteins and Cancer

Mutated versions of the three human Ras genes have been detected in ~30% of all human cancers, implying an important role for aberrant Ras protein function in carcinogenesis. For example, Ras gene mutations are highly prevalent in pancreatic (90%) (9), lung (30%) (607), and colorectal (50%) (59, 756) carcinomas. Because Ras proteins regulate diverse extracellular signaling pathways for cell growth, differentiation, and apoptosis, the deregulated function of other cellular components can cause
aberrant Ras protein function in the absence of mutations in the Ras genes themselves. Overexpression of ErbB2 or EGF receptor tyrosine kinase is common in breast cancers, and their transforming actions are dependent on signaling through the loss of negative Ras protein regulators (155). Similarly, the loss of function of negative Ras protein regulators, such as neurofibromin defective in type 1 neurofibromatosis-associated tumors, can cause aberrant upregulation of Ras protein function (42). Therefore, the importance of aberrant Ras protein function in human cancers may be greater than expected and may extend to tumors that do not harbor mutated Ras alleles.

H. Rap Proteins

The Rap subfamily consists of Rap1A, Rap1B, and Rap2. Rap1 proteins have been independently isolated by three laboratories by different methods: they have been isolated as homologs of Ras proteins by hybridization (573), they have been purified as small G proteins (smg p21) by column chromatography (337, 534), and they have been identified as K-Rev1 in a screen for cDNAs that revert the morphology of Ki-Ras-transformed cells (353). Interestingly, Rap1 proteins have an effector domain virtually identical to that of Ras proteins, suggesting that both proteins theoretically interact with similar effectors and show similar or antagonistic effects. The antagonistic function of K-Rev1 on Ras-transforming activity was the first studied (353). Rap1A binds to the two Ras-binding regions of Raf-1 (RBD and CRD), and this binding of Rap1A to CRD is competitive with Ras proteins (296, 297). Rap1 does not induce Raf-1 activation in intact cells but inhibits the Ha-Ras-induced Raf-1 activation in intact cells when Rap1 is overexpressed (124). However, most extracellular signals that induce Raf-1 activation, such as PDGF and EGF, activate rather than inhibit Rap1 (828). Furthermore, a phorbol ester induces Rap1 activation in Rat1 cells, but does not inhibit the PDGF- and EGF-induced activation of MAP kinase (828). These results suggest that the suppression of Ras protein function by Rap1 is simply due to the artificially competitive inhibition of the Ras protein binding to RBD or CRD.

In contrast to the role of Rap1 antagonistic to that of Ras proteins, evidence is accumulating that Rap1 functions independently of Ras protein signaling, utilizing effectors similar or identical to those of Ras proteins, like Raf proteins. Rap1, as well as Ki-Ras, induces DNA synthesis in Swiss 3T3 cells (11, 807). Rap1, as well as Ki-Ras, binds and activates B-Raf in vitro (541). In intact PC12 cells in response to cAMP and NGF, Rap1 is activated and induces B-Raf activation, causing sustained activation of the MAP kinase cascade that is necessary for neuronal differentiation (761, 806). Most recently, CD31, an important integrin adhesion amplifier, has been shown to selectively activate Rap1, but not Ha-Ras, R-Ras, or Rap2 (587). An activated mutant of Rap1 stimulates T lymphocyte adhesion to intercellular adhesion molecule and vascular cell adhesion molecule, as does C3G. Thus Rap1 regulates ligand-induced cell adhesion, and it may play a more general role in coordinating adhesion-dependent signals. In contrast to Rap1, little is known about Rap2 (573).

Several distinct second messenger pathways, including those for calcium (194), diacylglycerol (462), phospholipase C-γ (462), and cAMP (10), and perhaps others (497a), are able to induce Rap1 activation. Clearly, Rap1 activation is a common event, which suggests a function that is central in signal transduction processes. C3G is a Rap1-specific GEP containing a proline-rich domain that interacts with the SH3 domain of members of the Crk adaptor proteins, Crk I, Crk II, and Crk L (239, 355). In general, this association is constitutive, but tyrosine phosphorylation of Crk may disrupt the interaction (546). The SH2 domain of Crk binds directly to various activated receptor tyrosine kinases and phosphotyrosine-containing adaptor proteins (355). This association of Crk-C3G with these complexes may enhance GEP activity of C3G (301), suggesting that complex formation and dissociation of C3G regulate Rap1 activation by tyrosine kinases. However, in a human Jurkat T cell leukemia line, T-cell receptor-dependent induction of a Cbl-Crk L-C3G signaling complex does not activate Rap1 (586). Therefore, more work will be required to clarify how C3G complex formation is coupled to Rap1 regulation. Recently, two novel GEPs specific for Rap1, named Epac/cAMP-GEFI and nRap GEP/PDZ-GEF1/Hs-RA-GEF, have been identified (147, 148, 335, 402, 540). Epac/cAMP-GEFI has cAMP-binding and Ras GEP domains; thus this GEP activity is dependent on cAMP (148, 335). nRap GEP has been isolated as a binding partner of S-SCAM, that interacts with N-methyl-D-aspartate (NMDA) receptors and neurologin through PSD-95/Dlg-A/ZO-1 (PDZ) domains at synaptic junctions (540). In contrast to Epac/cAMP-GEFI, nRap GEP/PDZ-GEF1/Hs-RA-GEF has one PDZ, one Ras associated, and one Ras GEP domains as well as one COOH-terminal consensus motif for binding to PDZ domains. However, nRap GEP/PDZ-GEF1/Hs-RA-GEF has an incomplete cAMP-binding domain and its GEP activity is independent of cAMP. SPA-1, a Rap1 GAP, has been shown to interfere with Rap1 activation by membrane-targeted C3G (729). Overexpression of SPA-1 in HeLa cells suppresses Rap1 activation upon plating on dishes coated with fibronectin and results in the reduced adhesion. In addition, overexpression of SPA-1 in promyelocytic 32D cells also inhibited both activation of Rap1 and induction of cell adhesion by granulocyte colony-stimulating factor, suggesting that Rap1 is required for the cell...
adhesion induced by both extracellular matrix and soluble ligands (729).

I. Ral Proteins

The Ral subfamily consists of RalA and RalB (100, 101). Ral GDS, a Ral GEP, has been found to be a Ras protein effector (279, 342, 676). Moreover, insulin and EGF induce activation of Ral proteins, and this activation is inhibited by a dominant negative mutant of Ras proteins, suggesting that RalA is downstream of the Ras protein signaling pathway (783). In NIH 3T3 cells, both a dominant active mutant of Ha-Ras and Ral GDS synergize with Raf-1 in the induction of cell transformation and the activation of c-fos promoter (548, 741), and a dominant negative mutant of RalA inhibits the Ha-Ras- and Raf-1-induced transformation (741). These observations suggest that the Ral GDS-Ral protein pathway contributes to cell transformation and gene expression. However, a dominant active mutant of RalA alone cannot efficiently induce the oncogenic transformation or the c-fos induction compared with a dominant active mutant of Ha-Ras and Ral GDS (548, 741), suggesting that the transformation and the gene expression induced by Ral GDS may require other factors in addition to Ral proteins.

Three effectors for Ral proteins are known: RalBP1, phospholipase D, and filamin. RalBP1 contains a Rho GAP homology domain that exhibits the GAP activity for Rac/Cdc42 proteins, but not for Rho proteins (179). Although Rac/Cdc42 proteins contribute to the Ha-Ras-induced oncogenic transformation (582) (see below), it is unclear whether the association of Ral proteins with RalBP1 regulates the activity of these Rho/Rac/Cdc42 proteins. RalBP1 has been found to interact with POB1 and Reps1 (302, 791), which have proline-rich sequences responsible for interaction with Grb2 and Crk, and an Eps15 homology domain. Ral proteins are involved in endocytosis of the growth factor receptors probably through RalBP1, POB1, Eps15, and Epsin (511). Another Ral protein effector, phospholipase D, is also implicated in vesicle trafficking (179, 316). The activity of phospholipase D is induced by Src and Ras proteins. A dominant negative mutant of RalA inhibits both v-Src- and v-Ras-induced phospholipase D activity (316). The third effector protein of Ral is filamin (537). Either a dominant negative mutant of RalA or the RalA-binding domain of filamin blocks Cdc42-induced filopodium formation. A dominant active mutant of RalA elicits actin-rich filopodia, but it does not generate filopodia in filamin-deficient cells. Thus the Ral signaling appears to regulate vesicle trafficking, cytoskeletal organization, gene expression, and cell transformation. The GAP proteins for RalA were characterized and partially purified (48, 170); however, the molecular cloning of these proteins has not yet been achieved.

J. Other Ras Family Members

R-Ras has been shown to be involved in multiple biological functions: the ability to transform NIH 3T3 cells, the promotion of cell adhesion, and the regulation of apoptotic response in hematopoietic cells (128, 621, 695, 816). Unlike other Ras family members, R-Ras does not activate Raf proteins or MAP kinases in cells, whereas it stimulates PKB/Akt effectively through PI 3-kinase (444). TC21 has a highly oncogenic potential and is found mutated in some human tumors and overexpressed in breast cancer (37, 94, 300). As to the activation of Raf proteins by TC21, it is controversial (242, 611). Recently, new members of the Ras family, Rit and Rin, have been identified by an expression cloning screen (385). Rit is ubiquitously expressed, whereas Rin is expressed only in neural tissue. A unique feature of their structures is that they lack a known recognition signal for COOH-terminal prenylation. Nonetheless, both proteins localize on the plasma membrane, probably through a COOH-terminal cluster of basic amino acids. Rin binds calmodulin through a COOH-terminal motif, suggesting that Rin may be involved in calcium-mediated signaling in neurons (385). Rad is another member of Ras-like proteins that has originally been isolated as a gene overexpressed in the skeletal muscle of humans with type II diabetes (594). Kir/Gem has also been cloned as a gene that is overexpressed in cells transformed by abl tyrosine kinase (123) or cloned from mitogen-induced human peripheral blood T cells (429). Kir/Gem and Rad constitute a new family of Ras-related proteins. The distinct structural features of this family include the G3 GTP-binding motif, extensive NH2- and COOH-terminal extensions beyond the Ras-related domain, and a motif that determines membrane association (429). Rheb, another Ras protein-related molecule, has been isolated by differential cloning techniques to identify genes that are rapidly induced in brain neurons by synaptic activity (790). Expression of Rheb is rapidly and transiently induced in hippocampal granule cells by seizures and by NMDA-dependent synaptic activity (790). The amino acid sequence of Rheb is most closely homologous to yeast Ras1 and human Rap2. In the developing brain, Rheb mRNA is expressed at relatively high levels. Its close homology with Ras proteins and its rapid inducibility by receptor-dependent synaptic activity suggest that Rheb may play an important role in long-term activity-dependent neuronal responses (790). More recently, Ras protein-like proteins, named κB-Ras1 and κB-Ras2, have been identified (181). These proteins interact with IkBα and IkBβ, which are inhibitors for the nuclear transcription factor kappa B, NF-κB, and decrease the rate of degradation of IkBα compared with IkBβ (181).
IV. RHO/RAC/CDC42 PROTEINS AS REGULATORS OF BOTH CYTOSKELETAL REORGANIZATION AND GENE EXPRESSION

A. Outline

The mammalian Rho family consists of at least 14 distinct members as shown in Table 1 and Figure 1. The function of the Rho family was first demonstrated in yeast (5, 45, 320). Phenotypes of the mutants that carry mutations in these genes indicated that Rho/Cdc42 proteins are involved in the budding process, presumably through reorganization of the actin cytoskeleton (320, 798). In mammals, the function of Rac proteins was the first to be clarified. GTP-Rac1, in addition to two other cytosolic proteins, p47phox and p67phox, were shown to be required for the activation of NADPH oxidase of phagocytic cells (1, 2, 18, 358, 479, 649). Then, the function of mammalian Rho proteins was elucidated by use of an exoenzyme of Clostridium botulinum, named C3, that specifically ADP-ribosylates Rho proteins (6, 343, 512). C3 ADP-ribosylates an amino acid (Asn-41) in the effector region of RhoA and inhibits its function by preventing interaction with downstream effectors (651). By the use of C3, Rho proteins were first suggested to be involved in cytoskeletal control (97, 564). Rho proteins were subsequently shown to regulate formation of stress fibers and focal adhesions in fibroblasts by use of its dominant active mutant and Rho GDI (475, 600, 601) and to regulate Ca$^{2+}$ sensitivity of smooth muscle contraction (276). In contrast, Rac and Cdc42 proteins regulate formation of lamellipodia and filopodia, respectively (368, 522, 602). It has now been established that at least Rho/Rac/Cdc42 proteins regulate primarily cytoskeletal reorganization in response to extracellular signals in mammalian cells. Evidence has also accumulated that they may play additional roles in gene expression (126, 272, 473, 567, 692, 776). Furthermore, involvement of Rho/Rac/Cdc42 proteins in diverse cellular events, such as cell growth (341, 420, 552, 581–583, 794), membrane trafficking (4, 69, 86, 365, 380), development (227), and axon guidance (412) and extension (277, 314, 369), have been reported. In these cellular events, it is not known whether Rho/Rac/Cdc42 proteins directly regulate them or indirectly regulate them through cytoskeletal reorganization and gene expression. Many upstream regulators and downstream effectors have been identified for Rho/Rac/Cdc42 proteins, and although their modes of activation and action have gradually been elucidated, our understanding remains incomplete. Posttranslational modifications of Rho proteins are also crucial for their various functions including cell shape change, cell motility, cytoplasmic division of Xenopus embryo, and regulation of 1,3-β-glucan synthase of S. cerevisiae (305, 352, 475, 705).

B. Reorganization of the Actin Cytoskeleton

Reorganization of the actin cytoskeleton plays crucial roles in many cellular functions such as cell shape change, cell motility, cell adhesion, and cytokinesis. The actin cytoskeleton is composed of actin filaments and many specialized actin-binding proteins (671, 688, 826). Filamentous actin is generally organized into a number of discrete structures (Fig. 6): 1) stress fibers: bundles of actin filaments that traverse the cell and are linked to the extracellular matrix through focal adhesions; 2) lamellipodia: thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many migrating cells; membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substratum and fold backward; and 3) filopodia: fingerlike protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. They are found primarily in motile cells and neuronal growth cones. It is important, therefore, that the polymerization and depolymerization of cortical actin be tightly regulated. For the most part, this regulation of actin polymerization is orchestrated by Rho/Rac/Cdc42 proteins. Rho proteins regulate stress fiber formation (475, 600), while Rac proteins regulate ruffling and lamellipodia formation (602), and Cdc42 regulates filopodium formation (368, 522).

C. Rho/Rac/Cdc42 Protein Cycle: Cyclical Activation/Inactivation

The activation and inactivation of Rho/Rac/Cdc42 proteins are regulated by essentially the same mechanism as Ras proteins by GEPs and GAPs, respectively. However, they are further regulated by another class of regulator, GDIs (206, 275, 280, 704, 732). In the cytosol, Rho/Rac/Cdc42 proteins are complexed with the GDI and maintained in the GDP-bound inactive form. The GDP-bound form is first released from a GDI by a still unknown mechanism and is converted to the GTP-bound form by the action of a GEP. The GTP-bound form then interacts with the downstream effector(s). Thereafter, the GTP-bound form is converted to the GDP-bound form by the action of a GAP. The GDP-bound form then forms a complex with the GDI and returns to the cytosol.

Rho/Rac/Cdc42 proteins are posttranslationally modified with lipid as described above and therefore they have to be in complex with GDIs to remain soluble in the cytosol. However, it is unknown whether all the GDP-bound form of Rho/Rac/Cdc42 proteins are complexed with GDIs and remain in the cytosol. Some amount of the GDP-bound form may be associated with membranes, and it may be converted to the GTP-bound form and exert its function on the membrane. In this case, GDIs would not be essential for their cyclical activation and inactivation.
Many GEPs for Rho/Rac/Cdc42 proteins have been isolated and characterized as shown in Table 2 and numbers are still increasing. Most GEPs have been isolated as oncogenes. The GEPs thus far identified share a common motif, designated the Dbl-homology (DH) domain, for which the Dbl oncogene product is the prototype (93). Biochemical analysis has confirmed that DH domains of GEPs indeed show GEP activity on Rho/Rac/Cdc42 proteins in a cell-free assay system (259, 262, 468). In addition to the DH domain, GEPs share a PH domain, which may be involved in proper cellular localization presumably through interaction with PIP2 (469, 824). Some members of GEPs, such as Dbl and Vav1, have been shown to exhibit exchange activity in vitro for a broad range including Rho/Rac/Cdc42 proteins, whereas others appear to be more specific. Lbc, for example, and more recently discovered oncoproteins Lfc and Lsc are specific for Rho proteins (226, 823), whereas FGD1 and frabin are specific for Cdc42 (532, 738, 822). Although Vav1 is a GEP for Rac proteins (133, 245), Vav2, a GEP closely related to Vav1, functions preferably as a GEP for Rho proteins (642, 643). Some GEPs, such as Dbl, prefer the lipid-modified form of the substrate small G proteins to the lipid-unmodified ones (788).

In addition to the PH and DH domains, many GEPs have other domains that are commonly found in signaling molecules, such as the SH2 domain for Vav or the SH3 domain for Vav and Dbs, suggesting that they may have additional functions (93, 133, 642, 778). A GEP for Rho proteins, named p115 Rho GEF, that contains the regulator of G protein (RGS) domain has recently been identified (262, 367). RGS stimulates the intrinsic GTPase activity of the α-subunit of G12 and G13. p115 Rho GEF acts as an intermediary in the regulation of Rho proteins by Ga12 and Ga13 (260, 367). In addition, another Rho GEP (named PDZ-Rho GEF) that contains RGS and PDZ domains has been reported (204). These findings have provided a new model for a signaling pathway for Rho proteins from membrane receptors. Recently, SHP-2, a protein tyrosine phosphatase containing SH2 domains, has been demonstrated to suppress the activity of Vav2 and consequently to reduce the Rho’s ability to form stress fibers and focal adhesions (360). SHP-2 thereby positively regulates the hepatocyte growth factor (HGF)/scatter factor (SF)-induced cell scattering. However, detailed information regarding signaling cascades coupling the extracellular stimuli to activation of GEPs for Rac/Cdc42 proteins is still limited. Some GEPs like Tiam1 (248) and Ras GRF (667) carry a second PH domain. For Tiam1 and Ras GEF, this second, NH2-terminal PH domain mediates localization to cell membranes (74, 469). In addition to the PH domain, frabin has an actin-binding domain at its NH2-terminal region (532). The actin-binding domain in addition to the DH and first PH domains is essential for the filopodium formation mediated by frabin through Cdc42 (532, 738). Frabin furthermore induces
lamellipodium formation through indirect activation of Rac (553). The COOH-terminal FYVE and second PH domains, which associate with an unidentified membrane structure, in addition to the DH and first PH domains are necessary for this action (553).

The first GAP protein specific for Rho proteins was biochemically purified from human spleen and bovine adrenal gland (211, 212, 488). This protein, designated p50 Rho GAP, has GAP activity toward Rho/Rac/Cdc42 proteins in vitro (381). A number of proteins that exhibit GAP activity for Rho/Rac/Cdc42 proteins have subsequently been identified in mammalian cells (Table 2). These proteins all share a related GAP domain that spans ~140 amino acids of the protein but bears no significant resemblance to Ras GAP. The substrate specificity of Rho GAPs toward Rho/Rac/Cdc42 proteins varies with each GAP protein. Although some of these proteins exhibit GAP activity for several small G proteins in cell-free assay systems, their substrate specificities in vivo appear to be more restricted. For instance, the substrate spectrum of p50 Rho GAP in vitro encompasses Rho/Rac/Cdc42 proteins; however, in vivo, it appears to be restricted to Rho proteins only (603). Although first identified as a tyrosine-phosphorylated p120 Ras GAP-associated protein in Src-transformed cells and in growth factor-treated cells (169, 657), p190 Rho GAP was later shown to possess GAP activity for Rho proteins (656). Although the biological function of p190 Rho GAP is not well understood, the interaction of p190 Rho GAP with p120 Ras GAP has been suggested to induce a conformational change in p120 Ras GAP, resulting in increased accessibility of the effector binding surface of its SH3 domain (298). A role for p190 Rho GAP in regulating Rho protein function in cells undergoing cytoskeletal rearrangements has been suggested (95, 603), but it is not known whether this effect is induced by p190 Rho GAP as a downstream effector of Rho proteins.

Recent studies have shown that a cycle of inactivation and activation of Rho/Rac/Cdc42 proteins is necessary for dynamic cell functions such as growth factor-induced cell scattering. Expression of dominant active mutants of Rho/Rac/Cdc42 proteins inhibits HGF/SF-induced cell scattering (303, 331, 599), whereas C3 or Rho GDI blocks HGF/SF-induced cell scattering (706). The mode of action of Rho proteins in cell scattering remains to be clarified, but the Rho protein-regulated assembly and disassembly of stress fibers and focal adhesions have been suggested to be, at least in part, involved in this process (303, 331, 599, 706). It is not known how inactivation by GAPs is induced. In one case, integrin-induced formation of stress fibers inhibits Rho protein activation as part of a feedback inhibition system (593).

Rho GDI was originally isolated as a cytosolic protein that preferentially associated with GDP-RhoA and GDP-RhoB and thereby inhibited the dissociation of GDP (206, 732). Rho GDI requires the posttranslational lipid modifications of RhoA for its activity (286). Rho GDI prefers GDP-RhoA and GDP-RhoB to the corresponding GTP-bound forms and forms a ternary complex with the GDP-bound form (628, 732). Rho GDI is also capable of inhibiting GTP hydrolysis by Rho proteins (116, 261, 628), blocking both intrinsic and GAP-catalyzed GTPase activity. Rho GDI dissociates the GDP-bound form of prenylated RhoB from the membrane (308). Based on these properties of Rho GDI, it has been proposed that Rho GDI is involved not only in the regulation of the activation of Rho proteins but also in their translocation between the cytosol and the membrane (630, 702, 704). The GDP-bound forms of Rho proteins are complexed with Rho GDI and remain in the cytosol. When the GDP-bound form is released from Rho GDI, it is converted to the GTP-bound form by the action of Rho GEFs. The GTP-bound form then activates its specific downstream effector(s) until the GTP-bound form is converted to the GDP-bound form by Rho GAPs. Once the GDP-bound form is produced on the membrane, it is captured by Rho GDI and the complex returns to the cytosol.

Rho GDI has also been shown to be active not only on Rho proteins but also on Rac/Cdc42 proteins (18, 275, 391). In addition to Rho GDI, at least two other isoforms, named D4/Ly-GDI and Rho GDI-3, have been identified (390, 635, 809). Now, the originally identified Rho GDI is referred to as Rho GDIα or Rho GDI1; D4/Ly-GDI is named Rho GDIβ or Rho GDI2; and Rho GDI3 is named Rho GDIγ or Rho GDI3. Recent NMR studies have shown

**Table 2. Regulators of Rho/Rac/Cdc42 proteins**

<table>
<thead>
<tr>
<th>GEP</th>
<th>Substrate</th>
<th>Reference No.</th>
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<tr>
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<tr>
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<td>p115RhoGEF</td>
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<td>248</td>
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<td>FGID1</td>
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<tr>
<td>Pradin</td>
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<tr>
<td>GAP</td>
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<td>36, 381</td>
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<tr>
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<tr>
<td>D4/Ly-GDI</td>
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</tr>
<tr>
<td>RhoGDI-3</td>
<td>RhoB (not RhoA, RhoC)</td>
<td>809</td>
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that Rho GDIα has a pocket that masks the lipid moieties of Rho proteins (238), consistent with biochemical analyses (286). More recently, the X-ray crystallographic structure of the Cdc42/Rho GDI complex has revealed two major sites of interaction between Rho GDI and Cdc42 (280). The NH2-terminal regulatory region of Rho GDI binds to the switch I and II domains of Cdc42, leading to inhibition of both GDP dissociation and GTP hydrolysis. In addition, the geranylgeranyl moiety of Cdc42 inserts into a hydrophobic pocket within the immunoglobulin-like domain of the GDI molecule, keeping GDP-Cdc42 in the cytosol and permitting the dissociation of GDP-Cdc42 from membranes.

Although the mechanism by which Rho proteins are released from Rho GDI has largely been unknown, it has been shown that ERM, which consists of ezrin, radixin, and moesin, has the capacity to displace the GDP-bound form of Rho proteins from Rho GDIα (700). ERM has two functional domains: the NH2-terminal plasma membrane-binding and COOH-terminal F-actin-interacting domains (30, 730). ERM is translocated to the plasma membrane probably through the interaction with the cytoplasmic domain of integral plasma membrane proteins such as CD44, providing the F-actin binding sites. Rho GDIα interacts with the NH2-terminal fragments of ERM, and this interaction reduces Rho GDIα activity (700). The unfolding of ERM may induce Rho GDIα interaction with ERM NH2-terminal regions, causing release of the Rho-GDP and making it sensitive to the action of each Rho GEP (699, 700). The interaction of full-length ERM with the cytoplasmic fragment of CD44 does not induce the association of ERM with Rho GDIα (700). However, CD44 and Rho GDIα are coimmunoprecipitated with moesin (274). It is likely that full-length ERM unfold to permit interactions with Rho GDIα and CD44 via ERM NH2-terminal regions and F-actin via ERM COOH-terminal regions; a signal from CD44 may trigger this process. These findings suggest that the activation of Rho proteins is regulated in a temporally and spatially dynamic manner and is distinct from that of Ras proteins, which appears to be regulated in a unidirectional manner.

Little is known about the physiological function of Rho GDIs in vivo, but microinjection studies have shown that Rho GDIα inhibits several downstream functions of Rho proteins (303, 352, 475, 521, 630, 705, 706). Rho GDIα-deficient mice have revealed several abnormal phenotypes (722). These mice are initially viable, but they develop massive proteinuria mimicking nephrotic syndrome in humans, leading to death due to renal failure within a year. Histologically, degeneration of tubular epithelial cells and dilatation of distal and collecting tubules are readily detected in the kidneys. Rho GDIα-deficient mice are also infertile and show impaired spermatogenesis with vacuolar degeneration of seminiferous tubules. In contrast, Rho GDIβ-deficient mice show normal hematopoietic differentiation but subtle defects in superoxide production by macrophages derived from in vitro embryonal stem cell differentiation (244). Thus these two lines of evidence suggest that Rho GDIs play physiologically important roles.

Yeast Rom7/Bem4 is a novel type of Rho1- and Cdc42-related molecule (273, 419). This protein appears to be distinct from GEPs, GDIs, and GAPs, in the sense that it interacts genetically with the Rho1 pathway but does not show GEP, GDI, or GAP activity, and it is not a downstream effector.

D. Mode of Action of Rho Proteins in Cytoskeletal Reorganization

Mammalian Rho proteins are required for many actin cytoskeleton-dependent cellular processes, such as platelet aggregation, lymphocyte and fibroblast adhesion, cell motility, contraction, and cytokinesis (251, 704). In the yeast S. cerevisiae, Rho proteins, including Rho1, Rho2, Rho3, and Rho4, regulate budding and cytokinesis through reorganization of the actin cytoskeleton (80, 119, 200, 713). The mode of action of Rho proteins was first clarified for Rho1 in the yeast budding process. Pkc1, a yeast homolog of mammalian protein kinase C, was first shown to be a downstream effector of Rho1 (330, 525). Pkc1 regulates gene expression through the MAP kinase cascade, consisting of Bck1 (MAP kinase kinase kinase), Mkk1/Mkk2 (MAP kinase kinase), and Mpk1 (MAP kinase) (173, 396). This MAP kinase cascade regulates expression of the genes necessary for cell wall integrity. Bni1 is another effector of Rho1 (363). Bni1 also interacts with GTP-Rho3, GTP-Rho4 (T. Kamei and Y. Takai, unpublished observations), and GTP-Cdc42 (175). Bni1 has two domains, named formin homology (FH)1 and FH2 domains, which are found in a variety of proteins involved in cytoskeletal rearrangement needed to achieve cell polarity and cytokinesis (196). Bni1 binds the actin monomer-binding protein profilin via its FH1 domain to regulate reorganization of the actin cytoskeleton (175, 363). Furthermore, Bni1 binds Aip3 (Bud6), another actin-binding protein (15), and elongation factor 2, which is known to stimulate actin polymerization (739), suggesting that Bni1 is the downstream effector of Rho1 that directly regulates reorganization of the actin cytoskeleton. More recently, Bni1 has also been shown to participate in microtubule function, since disruption of BNII causes defects in spindle orientation (386), Kar9 localization (472), and growth defect together with mutation either PAC1 and NPI100 (199), whose gene products are implicated in microtubule function (215).

1,3-β-Glucan synthase is the third effector of Rho1 (157, 456, 580). This enzyme synthesizes 1,3-β-glucan, a major component of the cell wall. This series of experi-
ments has established that Rho proteins regulate complicated cell functions through multiple effectors in a cooperative manner. Another protein having FH domains has also been found in yeast and named Bnr1 (304). This protein serves as an effector of Rho4 and binds both profilin and Aip3 (Bud6), indicating that Rho4 regulates the actin cytoskeleton at least through Bnr1. Rho2 has 53% identity to Rho1, and a RHO2 null mutant does not show any growth defect (422). Hence, it has been assumed that Rho2 has redundant functions with Rho1, although several lines of evidence suggest Rho2-specific functions (149, 436). Rho3 and Rho4 are implicated in polarized growth presumably through regulating the actin cytoskeleton via their interactions with Bni1 and Bnr1 (175, 331a). Moreover, it has been shown that Rho3 interacts with Myo2 (type V myosin) and Exo70 (a component of the exocyst, a multiprotein complex which is involved in exocytosis) (606), suggesting the involvement of Rho3 in the polarized secretion.

Numerous downstream effectors of mammalian Rho proteins have been identified (Table 3). p160ROCK, also named ROKα/Rho kinase, is a recently identified, serine/threonine protein kinase (307, 395, 451) and a downstream effector of Rho proteins. The activity of this protein kinase is stimulated by GTP-Rho proteins. Many ROCK/Rho kinase substrates have been identified; these include the myosin binding subunit of myosin light-chain phosphatase (347), myosin light chain (13), ERM (202), and cofilin (425). Of these substrates, myosin light-chain phosphatase appears to be a physiological substrate (347). Guanosine 5'-O-(3-thiotriphosphate) (GTPγS), a poorly hydrolyzable GTP analog, increases the sensitivity of smooth muscle contraction to Ca2+ (354, 487, 785). This action of GTPγS is inhibited by C3 or an exoenzyme of *Staphylococcus aureus*, named EDIN, which also inhibits the function of Rho proteins, and it is mimicked by GTPγS-RhoA, but not by GDP-RhoA, indicating that Rho proteins are involved in GTPγS-induced Ca2+ sensitization of smooth muscle contraction (276). Subsequently, myosin light-chain phosphatase has been shown to be a downstream effector of Rho proteins (232, 524). ROCK/Rho kinase has finally been shown to phosphorylate and to inhibit myosin light-chain phosphatase, causing the sustained contraction of smooth muscle contraction even after decrease in Ca2+ concentrations (347). In this mode of action, Rho proteins do not induce smooth muscle contraction without Ca2+ triggering; rather, they modify Ca2+ sensitivity of smooth muscle contraction. In this sense, the physiological relevance of the ROCK/Rho kinase-induced direct phosphorylation of the myosin light chain and the subsequent activation of the myosin ATPase could not be considered (13). The physiological relevance of the phosphorylation of other substrate proteins, including moesin, is also controversial. ROCK/Rho kinase phosphorylates moesin, which induces microvilli formation (202), but another report indicates that ROCK/Rho kinase is not involved in this phosphorylation (452). p140mDia, a mammalian homolog of Bni1 and Bnr1 in the yeast *S. cerevisiae* (304, 363) and that of *Drosophila diaphanous* (92), has also been implicated as a downstream effector of Rho proteins (770). mDia has FH1 and FH2 domains. mDia as well as Bni1 and Bnr1 bind profilin via its FH1 domain to regulate reorganization of the actin cytoskeleton. Overexpression of mDia induces weak formation of stress fibers without affecting the formation of focal adhesions (510, 769). ROCK and mDia cooperatively regu-

![Table 3. Effectors of Rho/Rac/Cdc42 proteins](image)

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PI, phosphatidylinositol.
late the Rho protein-induced reorganization of the actin cytoskeleton (510, 769) (Fig. 6). ROCK is part of another downstream cascade of Rho proteins, a Rho-ROCK-LIM kinase pathway (425). ROCK directly phosphorylates LIM kinase, which in turn is activated to phosphorylate cofilin. Cofilin exhibits actin-depolymerizing activity that is inhibited as a result of its phosphorylation by LIM kinase (425, 693). Overexpression of LIM kinase induces the formation of actin stress fibers in a ROCK-dependent manner. Thus phosphorylation of LIM kinase by ROCK and subsequent increased phosphorylation of cofilin by LIM kinase contribute to the Rho protein-induced reorganization of the actin cytoskeleton. In addition to ROCK, a variety of Rho effector proteins have recently been discovered (Table 3). These include serine/threonine protein kinase PKN (14, 768), citron (424), rhoetkin (588), and rhophilin (768). Citron contains a protein kinase domain that is related to ROCK. Citron kinase localizes to the cleavage furrow and midbody of cultured cells (423). Overexpression of citron mutants results in the production of multinucleate cells, and a kinase-active mutant causes abnormal contraction during cytokinesis, suggesting that citron kinase is involved in the Rho protein-regulated cytokinesis (423).

E. Mode of Action of Rac/Cdc42 Proteins in Cytoskeletal Reorganization

The function of Rac/Cdc42 proteins in cytoskeletal reorganization was first demonstrated in the yeast. In the yeast S. cerevisiae, Cdc42 participates in recognition of the landmark that defines the incipient bud site (5, 320, 827). In fibroblasts, Rac proteins regulate formation of lamellipodia and membrane ruffles and subsequent stress fiber formation (602). In contrast, Cdc42 plays a key role in the formation of filopodia at the cell periphery followed by the formation of lamellipodia and membrane ruffles (300, 522). Both Rac/Cdc42 proteins induce the assembly of multimolecular focal complexes at the plasma membrane of fibroblasts (522). In addition, Rac/Cdc42 proteins regulate the cadherin-based cell-cell adhesion and hence control the fibroblast-mediated cell scattering of Madin-Darby canine kidney cells (284, 324, 331, 361, 624, 686, 707). Cdc42 may play a role in the polymerization of both actin and microtubules toward antigen-presenting cells (689). Furthermore, the involvement of Cdc42 in the control of cytokinesis in HeLa cells and Xenopus embryos has been reported (156, 164). In Drosophila, Rac/Cdc42 proteins are implicated in axonal guidance, wing hair formation, actomyosin-driven furrow canal formation, and nuclear positioning (130, 165, 214).

To date, several potential effectors of Cdc42 and Rac proteins have been identified (Table 3). Several of these proteins are common effectors for both Rac and Cdc42 proteins. Among them, the family of serine/threonine protein kinases known as PAKs are especially interesting. Homologs of the mammalian PAKs have been identified in S. cerevisiae (Ste20 and Cla4) (137, 384), S. pombe (Pak1, also called Shk1) (443, 556), Drosophila (PAK1) (258), and C. elegans (Ste20) (108). In the yeast S. cerevisiae, Cdc42 interacts with Ste20, which in turn associates with Ste5 and Bem1, both of which interact with actin (387, 821). Drosophila PAK1 also plays a role in dorsal closure (258). Thus it is likely that PAK proteins are involved in mediating the effect of Cdc42/Rac proteins on the cytoskeleton. Most recently, Drosophila Trio, a GEP for Rac proteins, has been shown to play an important role in axon guidance (32, 43, 403, 515). One of the two Trio GEP domains is critical in photoreceptor axon guidance (515). This GEP domain activates Rac, which in turn activates Pak. Trio interacts genetically with Rac proteins, Pak, and Dock, an SH2-SH3 docking protein. Thus Trio regulates Rac proteins, which subsequently activates Pak, linking guidance receptors to the growth cone cytoskeleton (515). However, in mammalian cells, the role of PAKs remains unclear (Fig. 6B). Expression of a mutant form of Rac or Cdc42 protein that is unable to bind and activate PAKs can still induce the formation of membrane ruffles and lamellipodia (323, 379), indicating that PAKs are not essential for the Rac protein-elicited membrane ruffling and lamellipodium formation or for the Cdc42-triggered filopodium formation. This does not exclude the possibility, however, that PAKs themselves may play a role in cytoskeletal rearrangement by inducing actin reorganization independently of Rac/Cdc42 proteins. Alternatively, PAKs may mediate effects on the cytoskeleton induced by Rac/Cdc42 proteins, which are different from the immediate actin reorganization (437, 653).

All PAKs identified to date share a similar 18-amino acid CRIB (Cdc42/Rac interactive binding) motif that mediates the interaction with Rac/Cdc42 proteins. Most recently, N-WASP, a ubiquitously expressed Cdc42-interacting protein (470), and the Arp2/3 complex have been shown to participate in the downstream cascade of Cdc42-induced actin polymerization (49, 417, 418, 610, 697, 775, 782) (Fig. 6C). Wiskott-Aldrich syndrome protein (WASP), which is only expressed in hematopoietic cells, was originally identified as a protein mutated in patients with Wiskott-Aldrich syndrome (31, 698). WASP and N-WASP (470) possess a PH domain that binds PI(3) and a CRIB domain. The binding of both GTP-Cdc42 and PI(2) to N-WASP activates N-WASP by stabilizing the active conformation of this molecule. The COOH terminus of N-WASP binds the Arp2/3 complex and consequently stimulates its ability to nucleate actin polymerization in vitro (49, 415, 417, 418, 610, 697, 775, 782). Therefore, the interaction of N-WASP with the Arp2/3 complex is a core mechanism that directly connects the Cdc42-mediated...
have also been shown to activate the transcription factor activate serum response factor (SRF) (272, 776). They activate Rac proteins in JNK regulation (227).

of dorsal closure in endogenous Rac/Cdc42 protein activity. Genetic analysis physiological activation of the JNK pathway is induced by however, unclear, and it is still largely unknown whether Rho/Rac/Cdc42 proteins, such as Dbl (473). The exact role effects have been observed with oncogenic GEPs for JNK and p38 activities (126, 473). Furthermore, the same expression of a dominant active mutant of Rac proteins regulate the activation of Jun NH2-terminal kinases (JNK) and p38 MAP kinase (126, 473). Expression of a constitutively active mutant of Rac or Cdc42 proteins in HeLa, NIH 3T3, and COS cells results in a stimulation of JNK and p38 activities (126, 473). Furthermore, the same effects have been observed with oncogenic GEPs for Rac/Cdc42 proteins, such as DbI (473). The exact role of Rac/Cdc42 proteins in MAP kinase activation is, however, unclear, and it is still largely unknown whether physiological activation of the JNK pathway is induced by endogenous Rac/Cdc42 protein activity. Genetic analysis of dorsal closure in Drosophila also supports a role for Rac proteins in JNK regulation (227).

Rho/Rac/Cdc42 proteins have also been reported to activate serum response factor (SRF) (272, 776). They have also been shown to activate the transcription factor NFkB (567, 692). In addition, generation of reactive oxy-

gen species by Rac proteins might be the trigger for NFkB activation. This observation may be related to the fact that Rac proteins regulate an NADPH oxidase enzyme complex in phagocytes to produce superoxide as described earlier (1, 2, 18, 358, 479, 649). Rho/Rac/Cdc42 proteins are required for G1 cell cycle progression (420, 552, 794), but it is unclear whether this is due to their effects on the actin cytoskeleton and integrin adhesion complexes or, instead, to more direct effects on gene transcription.

G. Other Functions of Rho/Rac/Cdc42 Proteins

Various studies have suggested the involvement of Rho/Rac/Cdc42 proteins in membrane-trafficking processes. Cytoskeletal rearrangements are closely coupled to the onset of phagocytosis. Rho/Rac/cdc42 proteins are implicated in one or more steps of the phagocytic response (86, 129, 449). Two distinct mechanisms for the phagocytic response have recently been defined (8, 514). In type I phagocytosis, plasma membrane protrusions extend to engulf the particle and drag it into the cell; this is mediated by coordinated actions of Rac/Cdc42 proteins (86). In type II phagocytosis, particles sink into actin-lined investigations in the plasma membrane; here, internalization is dependent on RhoA (86). There are morphological similarities between these processes and the invasion of mammalian cells by certain pathogenic bacteria. Shigella invasion starts with actin nucleation and Rho protein-induced actin polymerization (4, 496). This is followed by continued actin polymerization around membrane-bound protrusions that fold over the bacterium and coalesce to engulf it. The complete inhibition of Shigella-induced membrane folding by C3 suggests that actin polymerization is essential for the generation of the surface extensions (4, 496). Interestingly, Cdc42 has been shown to play a direct role in Salmonella internalization (107) and in Shigella flexneri motility (696).

Rho/Rac proteins have also been implicated in the regulation of endocytosis. In mammalian cells, expression of dominant active mutants of Rho/Rac proteins reduces the efficiency of endocytosis of the transferrin receptor (380), and a dominant active mutant of RhoA also blocks internalization of muscarinic acetylcholine receptors (757). Expression of a dominant active mutant of RhoA causes an increase in clathrin-independent endocytosis in Xenopus oocytes (639). In Swiss 3T3 cells, expression of a dominant active mutant of Rac proteins stimulates pinocytosis (602), and the pinocytic vesicles are coated with the Rac signaling partner PAK1 (150). However, expression of a dominant active Rac protein has no effect on pinocytosis in baby hamster kidney cells (398); thus the role of Rac proteins in pinocytosis is still unclear.

Rho/Rac proteins have also been implicated in the
regulation of secretory vesicle transport (69, 555a). In
mast cells, Rho/Rac proteins stimulate the exocytosis of
secretory granules, whereas C3 and dominant-negative
mutants of Rho/Rac proteins inhibit GTP$\gamma$S-induced se-
cretion (526). In PC12 cells, RhoA, Rac1, and Rho GDI are
also involved in Ca$^{2+}$-dependent exocytosis at least partly
through the reorganization of actin cytoskeleton, and the
activation of RhoA or Rac1 alone is not sufficient for this
reaction (365). RhoD and RhoB are localized to endocytic
vesicles, suggesting that they might perform roles in the
regulation of intracellular trafficking (209, 498).

Rho/Rac/Cdc42 proteins have been implicated in
morphogenesis by regulation of cytoskeletal rearrange-
ments. For instance, the Drosophila homologs of Rac1,
Rac2, and Cdc42, namely, DRac1, DRac2, and DCdc42,
respectively, are highly expressed in the nervous system
and in the mesoderm during development, respectively
(411, 412). Particularly, the inhibition of axonal outgrowth
induced by a dominant active or dominant negative mu-
tant of DRac1 is due to different cytoskeletal defects in
developing neurons, suggesting that cyclical activation
and inactivation of DRac1 is crucial for axonal develop-
ment (214, 412). In addition, expression of a dominant
active mutant of DCdc42, DCdc42V12, in the neurons of
fly embryos results in a similar but qualitatively differ-
ent effect on neuronal development, compared with
DRac1V12 (412). In mammalian N1E-115 neuroblastoma
cells, Rho proteins appear to inhibit neurite outgrowth,
whereas Rac/Cdc42 proteins promote neurite outgrowth,
previously by regulating the formation of growth cone
filopodia and lamellipodia (277, 369). In contrast, in chick
dorsal root ganglion neurons, Rho proteins induce neurite
outgrowth, while an activated mutant of Rac proteins
increases the proportion of collapsed growth cones (317).
DRac1 and DCdc42 have also been implicated in muscle
differentiation (172, 412). Rac/Cdc42 proteins also regu-
late muscle development, probably via their effects on
membrane fusion and the actin cytoskeleton. Finally, lit-
tle is known at present about the role of Rho proteins in
neuronal and muscle development. In summary, Rho/Rac/
Cdc42 proteins have multiple essential functions during
morphogenesis.

V. RAB PROTEINS AS REGULATORS
OF VESICLE TRAFFICKING

A. Outline

Rab proteins exist in all eukaryotic cells and form the
largest branch of the small G protein superfamily (103,
447, 528, 529, 550, 636). The yeast S. cerevisiae genome
sequence encodes 11 Rab proteins (383). In mammalian
cells, over 50 Rab proteins (including isoforms) are
known (447, 528, 529, 550, 636). Evidence that Rab pro-
teins regulate intracellular vesicle trafficking was first
obtained genetically in the yeast S. cerevisiae. Many
genesis essential for secretion were isolated and named the
SEC genes (527). One of the SEC genes, the SEC4 gene,
was shown to encode a small G protein that is required for
vesicle trafficking from the Golgi apparatus to the plasma
membrane (623). The yeast YPT1 gene was first discov-
ered as a gene located between the actin and tubulin
genes in 1983 (208) and was later identified to encode a
small G protein (641). Ypt1 was shown to be later in-
volved in vesicle trafficking from the endoplasmic reticu-
lim to the Golgi apparatus (33, 650). Subsequently, a large
body of evidence has accumulated in support of a role for
Rab proteins in vesicle trafficking from yeast to human
(447, 528, 529, 550, 636).

B. Vesicle Trafficking

Transmembrane proteins and secreted, soluble pro-
teins are transported from one membrane compartment
to another by vesicles (465, 579, 616) (Fig. 7). Newly
synthesized secretory proteins are translocated into the
endoplasmic reticulum and are then transported to the
plasma membrane via the Golgi apparatus by vesicles;
some proteins are delivered to prelysosomes. In parallel,
macromolecules that are taken up from the plasma mem-
brane are transported inward to endosomes and lysos-
omes by vesicles. Some cell-surface proteins including
receptors for extracellular ligands transit through a reacy-
lcing endosome and are recycled back to the plasma
membrane. Thus exocytosis, endocytosis, and recycling
are performed by intracellular vesicle trafficking. There
are two exocytotic pathways: regulated and constitutive
pathways. In the regulated pathway, secretory proteins
are stored at very high concentrations in the core of

![FIG. 7. Principal mechanism of intracellular vesicle trafficking. Rab,
Rab proteins; Sar1/Arf, Sar1/Arf proteins.](image-url)
secretory granules that can be mobilized by an exocytosis pathway in response to neural or hormonal stimuli. In contrast, constitutive exocytosis occurs independently of extracellular stimuli and proceeds at a steady rate. Constitutive exocytosis is responsible for the insertion of plasma membrane proteins and secretion of extracellular proteins, such as matrix components and plasma proteins. Vesicle trafficking is needed for various other cell functions, such as establishment of cell polarity, cytokinesis, and cell motility.

There are at least four principal events in a given step of intracellular vesicle transport: 1) budding of a vesicle from the donor membrane; 2) targeting of the vesicle to the acceptor membrane; 3) docking of the vesicle to the acceptor membrane; and 4) fusion of the vesicle with the acceptor membrane (Fig. 7). Rab proteins regulate these processes (447, 528, 529, 550, 636). Substantial evidence has accumulated that most Rab proteins regulate the targeting/docking/fusion processes and that some of them regulate the budding process, which is mainly regulated by Sar1/Arf proteins (see sect. vi).

There are two types of Rab proteins: one type is involved in regulated secretion, and the other type is involved in other vesicle transport steps and is essential for this function (447, 528, 529, 550, 636). Of the first type, the mode of action of Rab3A has most extensively been investigated (703). Rab3A plays a key regulatory role in Ca\(^{2+}\)-dependent exocytosis, particularly in neurotransmitter release from nerve terminals. Studies on Rab3A-deficient mice have revealed that Rab3A is not essential for basal neurotransmission but modulates it, thereby contributing to synaptic plasticity (91, 216, 217, 218, 408).

In contrast, mutations of Rab proteins mediating other vesicle transport steps can have more dramatic consequences. In the yeast S. cerevisiae (383), Rab mutants are often characterized by a massive accumulation of the vesicles in the respective secretory pathways (623). Moreover, some of them are essential for cell viability.

FIG. 8. Subcellular localization of Rab proteins. Plain, localization of each Rab protein; italic, functioning site of each Rab protein.
C. Localization of Rab Proteins

Rab proteins contain unique, hypervariable COOH-terminal domains with two Cys residues, both of which require geranylgeranylation by geranylgeranyltransferase type II as described above. This lipid modification is essential for the membrane association of Rab proteins (27, 322, 340, 349, 568, 765). The COOH-terminal hypervariable region is important for the correct targeting of Rab proteins to their target membranes (64, 102), but how it directs localization remains to be clarified.

At steady state, 10–50% of a given Rab protein is detected in the cytosol. This pool is maintained in the GDP-bound form through interaction with Rab GDIs. Each Rab protein is localized at organelles and is involved in the vesicle transport of the organelle (447, 528, 529, 550, 636) (Fig. 8). For example, Rab1, Rab2, and Rab6 are localized at the endoplasmic reticulum and the Golgi apparatus and regulate vesicle transport along the biosynthetic/secretory pathway (104, 240, 265, 448, 575, 720); Rab3 is localized on secretory granules including synaptic vesicles and is involved in Ca\(^{2+}\)-dependent exocytosis (91, 140, 183–185, 216, 217, 282, 319, 408, 476, 477, 625); Rab4 and Rab5 are present on early endosomes and regulate early steps of the endocytic process, mediating endosome-endosome fusion and receptor recycling, respectively (73, 104, 237, 747, 748). However, the sites of action of the majority of Rab proteins have not yet been elucidated. In addition, it seems likely that some functional specialization of Rab proteins takes place, both in different cell types and in different organisms. One example is Rab11A and Rab11B and its two yeast homologs Ypt31p and Ypt32p. A role for Rab11 has been documented in late recycling of transferrin receptors (590, 736) and in trans-Golgi network-to-plasma membrane transport (109). Yeast Dss4 and its mammalian counterpart, Mss4, were first isolated and characterized as Rab GEPs (79, 497). Dss4 is active on both Sec4 and Ypt1 and Mss4 is active on a subset of Rab proteins. Moreover, the exchange activities of both GEPs are very low compared with the values of other GEPs for Ras and Rho/Rac/Cdc42 proteins. These proteins are unlikely to serve as physiologically relevant GEPs, and their real roles are currently unknown. Only three physiological Rab GEPs have been isolated and characterized: Rab3 GEP, specific for the Rab3 subfamily members (762); Rabex-5, specific for the Rab5 subfamily members (290); and Sec2 for Sec4 (763). Rab3 GEP is ubiquitously expressed but abundant in brain (762). It is active at least on Rab3A, Rab3C, and Rab3D and is inactive on other Rab proteins, including

D. Rab Protein Cycle: Cyclical Activation/Inactivation and Translocation

Rab proteins cycle between the GDP-bound inactive and GTP-bound active forms and between the cytosol and the membranes, and these cyclical activation, inactivation, and translocation are regulated by at least three types of regulators: GEPs, GDIs, and GAPs (447, 528, 703) (Fig. 9). In the cytosol, a Rab protein is maintained in the GDP-bound inactive form by a GDI. The GDP-bound form is first released from the GDI by a still unknown mechanism that is coupled to the delivery of the Rab protein to a specific membrane compartment (673, 735); the Rab protein is subsequently converted to the GTP-bound form by the action of a GEP. The GTP-bound form then interacts with a downstream effector(s). Thereafter, the GTP-bound form is converted to the GDP-bound form by the action of a GAP. The GDP-bound form produced on the membrane then complexes with the GDI and returns to the cytosol as a Rab protein-Rab GDI complex. Thus a characteristic of Rab proteins is that a cycle of membrane association/dissociation is superimposed onto their GDP/GTP cycle.

Yeast Dss4 and its mammalian counterpart, Mss4, were first isolated and characterized as Rab GEPs (79, 497). Dss4 is active on both Sec4 and Ypt1 and Mss4 is active on a subset of Rab proteins. Moreover, the exchange activities of both GEPs are very low compared with the values of other GEPs for Ras and Rho/Rac/Cdc42 proteins. These proteins are unlikely to serve as physiologically relevant GEPs, and their real roles are currently unknown. Only three physiological Rab GEPs have been isolated and characterized: Rab3 GEP, specific for the Rab3 subfamily members (762); Rabex-5, specific for the Rab5 subfamily members (290); and Sec2 for Sec4 (763). Rab3 GEP is ubiquitously expressed but abundant in brain (762). It is active at least on Rab3A, Rab3C, and Rab3D and is inactive on other Rab proteins, including
Rab2, Rab5A, Rab10, and Rab11. It prefers the lipid-modified form to the lipid-unmodified form as a substrate. A \textit{C. elegans} counterpart gene of this mammalian GEP, \textit{Aex-3}, has been identified genetically; mutation of this gene causes a defect in synaptic transmission (310). Rabex-5 forms a tight complex with Rabaptin-5, a Rab5 effector, and activation of Rab5 by Rabex-5 is likely coupled to effector recruitment (290). Rabex-5 is homologous to Vps9, a yeast protein involved in endocytosis (77). In the yeast, Sec2, a GEP for Sec4, is genetically essential for the Golgi-to-plasma membrane transport (763). Sec2 shares sequence homology to Rabin-3 that specifically interacts with a specific GEP. It is not known whether Rabex-5 and Sec2 require lipid modifications of their small G protein substrates. Only two GAPs for Rab proteins have been isolated and characterized in mammalian cells: Rab3 GAP, specific for the Rab3 subfamily members (205, 501) and GAPCenA, specific for Rab6 (135). Rab3 GAP is ubiquitously expressed and enriched in the soluble synaptic fraction in brain (205, 501, 543). Rab3 GAP consists of two subunits, a catalytic and a noncatalytic subunit (205, 501). It is active at least on Rab3A, Rab3B, Rab3C, and Rab3D and is inactive on other Rab proteins, including Rab2, Rab5A, and Rab11 (205). It prefers the lipid-modified form to the lipid-unmodified form as a substrate. The role of the noncatalytic subunit of Rab3 GAP is unknown, but Sar1 GAP also consists of catalytic (Sec23) and noncatalytic (Sec24) subunits (270, 808) (see sect. vi). GAPCenA is ubiquitously expressed (135). It is mainly cytosolic, but a minor pool is associated with the centrosome. It forms a complex with \(\gamma\)-tubulin and may play a role in microtubule nucleation. In the yeast \textit{S. cerevisiae}, five GAPs have been isolated and characterized: Gyp1 (GAP for Sec4 and Ypt1), Gyp6 (GAP for Ypt6), Gyp7 (GAP for Ypt7), Mdr1p/Gyp2p (GAP for Ypt6 and Sec4), and Msb3p/Gyp3p (GAP for Sec4, Ypt6, Ypt51, Ypt31/Ypt32, and Ypt1) (7, 163, 691, 760). Interestingly, Gyp proteins and GAPCenA (but not Rab3 GAP) share significant homology to yeast cell cycle checkpoint proteins, Bub2 and Cdc16, raising a possibility that these Rab GAPs serve to coordinate membrane trafficking with other events taking place during mitosis, such as the control of microtubule nucleation. GAPs terminate the function of Rab proteins, but it is not yet clear whether GTP hydrolysis is important for Rab proteins to accomplish their functions. The cycle of GTP hydrolysis of Rab5 has been shown to be uncoupled to the Rab5-regulated endosome-endosome fusion (619). Similarly, the GTP hydrolysis of Ypt1 seems dispensable for the Ypt1-mediated fusion events in the yeast (598). In these cases, the GTP-bound form seems to be requisite or even stimulatory for the progression of a vesicle toward membrane fusion. In contrast, for Rab3A, the GTP-bound form might interact with a prefusion complex, thus preventing fusion. The GTPhase-deficient mutant of Rab3A inhibits Ca\(^{2+}\)-dependent exocytosis from PC12 cells and chromaffin cells (282, 319) (see below). In this case, Rab3 GAP plays a crucial role in the function of Rab3 proteins. Three isoforms of Rab GDI have been isolated: Rab GDI\(\alpha\) (Rab GDI-1), Rab GDI\(\beta\), and Rab GDI\(\gamma\) (Rab GDI-2)(315, 453, 520, 629, 666). Southern blot analysis of genomic DNA indicates that both mouse and rat contain at least five Rab GDI genes (315). In contrast, there is only one Rab GDI gene (RDI1) in the yeast \textit{S. cerevisiae}, and it is essential for cell viability (213). The properties of Rab GDI\(\alpha\) and Rab GDI\(\beta\) have most extensively been studied (26, 27, 28, 315, 520, 629, 665, 666, 800). Rab GDI\(\beta\) is ubiquitously expressed, whereas Rab GDI\(\alpha\) is abundantly expressed in neuronal cells (520). Despite the different tissue distribution, both isoforms show similar biochemical properties (26, 28, 520, 629, 800). 1) Both proteins bind the lipid-modified, GDP-bound form, but not any other forms such as the lipid-modified, GTP-bound form, the lipid-unmodified GDP-bound form, and the lipid-unmodified GTP-bound form. 2) Both proteins inhibit the basal and GEP-stimulated dissociation of GDP from the GDP-bound form. 3) Both proteins inhibit the binding of the GDP-bound form to the membrane and keep it in the cytosol. 4) When the GDP-bound form is produced from the GDP-bound form on the membrane, both proteins bind the GDP-bound form and retrieve it from the membrane. In addition to these activities, Rab GDI plays an important role in specific delivery of Rab proteins to their target membranes (572, 673, 703, 735). For instance, Rab5-Rab GDI and Rab9-Rab GDI complexes mediate in vitro binding of Rab5 and Rab9 to organelles where these proteins normally reside (673, 735). The GDP/GTP exchange reaction does not seem to be a prerequisite for the Rab protein binding to the membranes, because the GDP-bound form can be transiently detected in the membranes after delivery of Rab protein-Rab GDI complexes onto membranes is likely to be followed by GDP/GTP exchange and the release of the GDI. A GDI dissociation factor (GDF) has been identified that is active on Rab5 and Rab9 and devoid of GEP activity but is not yet fully characterized (154). Rab GDI\(\alpha\) and Rab GDI\(\beta\) are significantly related to the mammalian \textit{Chorodieremia} gene product CHM, which delivers Rab proteins to the catalytic subunit of geranylgeranyltransferase II as described above (645). \textit{Chorodieremia} is an X-linked form of retinal degeneration (267). Recently, the crystal structure of Rab GDI\(\alpha\) has been determined, and the sequence-conserved regions are clustered on one face of the molecule (633). The two most sequence-conserved regions, which form a compact structure at the apex of Rab GDI\(\alpha\), play a crucial role in the binding of Rab proteins. Mutations of this region in the
human Rab GD1α gene are responsible for X-linked non-specific mental retardation (see sect. vG) (138).

E. SNAREs and Tethering Proteins in Vesicle Targeting/Docking/Fusion

According to the SNARE hypothesis, there are vesicle and target membrane SNAREs (v- and t-SNAREs) and these two types of SNAREs interact with each other, resulting in the docking of vesicles with their target membranes (615) (Fig. 10). Recent results suggest that SNARE pairing cannot be solely responsible for the appropriate targeting and docking of transport vesicles. For example, the docking of endoplasmic reticulum-derived vesicles with Golgi membranes can occur in the absence of SNARE components (83). Similarly, initial events leading to the homotypic fusion of vacuoles are not dependent on SNARE components (632, 740). Moreover, SNARE components can form promiscuous complexes in vitro (799). These results suggest that SNAREs are not primarily involved in the specific targeting of vesicles.

Evidence is accumulating that targeting/docking specificity is determined by the collaboration of several factors. Tethering proteins play key roles in vesicle targeting/docking (571, 771). This group includes Uso1 (83), TRAPP (620), p115 (505, 620), exocyst (246), and EEA1 (115), all of which bind membranes before the formation of SNARE complexes. In S. cerevisiae, Uso1 and a large protein complex named TRAPP are implicated in endoplasmic reticulum-to-Golgi transport (83, 620, 749); the exocyst, a large protein complex consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, and Exo70, is involved in targeting of post-Golgi vesicles to the plasma membrane (246). A mammalian version of the exocyst has been identified in mammalian cells, where it may be involved in targeting of vesicles (or docking with) the basolateral membrane of epithelial cells (243a, 295). In mammalian cells, a complex of p115, GM130, and giantin induces the interaction of Golgi-derived vesicles with Golgi membranes (505, 675), and EEA1 is involved in the homotypic endosome fusion process (115). EEA1 has recently been found to be an effector of Rab5 (115) (see below). It is likely that the events that precede stable, SNARE-dependent docking of vesicles or membranes are the result of a network of interactions between many proteins including tethering proteins. Although the direct interaction between Rab proteins and the tethering proteins has only been documented for Rab5/EEA1, Sec4, and the exocyst, Rab proteins are likely candidates for orchestrating vesicle targeting through tethering proteins as described below.

F. Mode of Action of Rab Proteins in Vesicle Targeting/Docking/Fusion

Several Rab protein effectors have been isolated and characterized. The first effector to be identified is Rabphilin-3 for the Rab3 subfamily members (662, 663). Rabphilin-3 is expressed in neurons and interacts preferentially with GTP-Rab3A. Rabphilin-3 is present on the surface of synaptic vesicles (478). It has not been established how Rabphilin-3 localizes to synaptic vesicles: one model is that it is recruited to the vesicles by Rab3A, analogous to the Ras protein-Raf protein kinase system (397, 683); another model is that it is targeted to synaptic vesicles independently of Rab3 (460, 654, 664). The NH2-terminal region constitutes the Rab3-binding region; the COOH-terminal region contains two C2 domains that bind Ca2+ and phospholipid (566, 662, 792). Expression or microinjection of either the NH2-terminal or the COOH-terminal region of Rabphilin-3 blocks Ca2+-dependent exocytosis in several different systems (78, 117, 364). Recently, it was shown that the abnormalities of synaptic transmission and synaptic plasticity that are observed in Rab3A-deficient mice are not observed in Rabphilin-3-deficient mice (638). This observation suggests that another Rab3 effector is present and compensates for loss of function of Rabphilin-3 in these mice. Rim is another effector of Rab3 proteins (766). Rim has an NH2-terminal Rab3A-binding domain that is homologous to that of Rabphilin-3, a cen-

Rabaptin-5 also interacts with the NH2-terminal region of terminal Rab5-binding domain. The NH2 terminus of Rabaptin-5 has an NH2-terminal coiled-coil region that overexpression of a GTPase-deficient mutant of Rab5. In addition, its overexpression induces the formation of large endosomes, a phenotype that is also observed upon addition, its overexpression induces the formation of the cytosol to endosomal membranes by GTP-Rab5. In contrast to Rab3A and Rabphilin-3, Rim is clearly absent from synaptic vesicles but enriched on the presynaptic plasma membrane, especially at the active zone. Although the precise function of Rim is still obscure, overexpression of complementarity-determining regions, which are potentially involved in the interactions of Rab proteins with their effectors.

Thus several effectors have been identified within the past 5 years. In addition, the recent determination of the X-ray crystal structure of Rab3A complexed to the Rab3-binding domain of Rabphilin-3 has enabled the identification of SNARE complexes (Fig. 10). Rab proteins are not core components of SNARE complexes. However, genetic studies in yeast have shown that functions of Rab and SNARE proteins are linked: the effects of a mutation in the effector domain of Sec4 is suppressed by overexpression of Sec9, the SNAP-25-like protein (63), and Ypt1 is involved in the priming of t-SNARE (401). In this process, Rab proteins may function to recruit tethering proteins onto membranes and coordinate loose membrane tethering to induce the SNARE complex-mediated, tighter and stable docking process.

GTP-Sec4 interacts with Sec15, a component of the exocyst (246). Then, a chain of protein-protein interactions leads to the assembly of SNARE complexes (Fig. 10). Rab proteins are not core components of SNARE complexes. However, genetic studies in yeast have shown that functions of Rab and SNARE proteins are linked: the effects of a mutation in the effector domain of Sec4 is suppressed by overexpression of Sec9, the SNAP-25-like protein (63), and Ypt1 is involved in the priming of t-SNARE (401). In this process, Rab proteins may function to recruit tethering proteins onto membranes and coordinate loose membrane tethering to induce the SNARE complex-mediated, tighter and stable docking process.
EEA1, interacts not only with an effector of Vps21 (a yeast Rab protein), but also with Vps45, a member of the Sec1/Munc18 family (569). In addition, other docking complexes regulated by Rab proteins have been characterized. For example, Rabphilin-3 and Rim, two effectors of Rab3 proteins, may be part of a complex that links synaptic vesicles to the presynaptic plasma membrane in neurons.

G. Rab3A in Ca\(^{2+}\)-Dependent Exocytosis

Transient overexpression or microinjection of a dominant active mutant of Rab3A inhibits Ca\(^{2+}\)-dependent secretion from bovine chromaffin cells and PC12 cells as described above (282, 319). In contrast, Rab3B stimulates secretion: in antisense RNA experiments performed in anterior pituitary cells, reduction in Rab3B mRNA inhibited Ca\(^{2+}\)-dependent secretion from these cells (407), and when a dominant active mutant of Rab3B was stably expressed in PC12 cells, where endogenous Rab3B is absent, Ca\(^{2+}\)-dependent norepinephrine secretion was markedly stimulated (772). The explanation for these apparently opposite effects of Rab3A and Rab3B on Ca\(^{2+}\)-dependent exocytosis is currently unknown.

Studies on Rab3A-deficient mice have revealed an important insight into Rab3A function. Synaptic depletion is much faster in the hippocampal CA1 region of these mice, although two forms of short-term synaptic plasticity, paired-pulse facilitation and posttetanic potentiation, are unaffected (216). These findings suggest that Rab3A plays a role in the recruitment of synaptic vesicles for exocytosis. In Rab3A-deficient, cultured hippocampal cells, Rab3A is involved in a late step in vesicle fusion (217). Moreover, Rab3A-deficient mice show reduced formation of postsynaptic long-term potentiation in the CA3 region (91). Thus Rab3A is not essential for basal synaptic transmission; rather, it modulates synaptic vesicle trafficking, thereby contributing to synaptic plasticity.

Recent genetic analysis of X-linked, nonspecific, mental retardation has revealed that mutations or deletions of the Rab GDI\(\alpha\) gene can cause this disease (138). Notably, Rab GDI\(\alpha\) is localized to the distal part of chromosome Xq28. Mutations in this region have recently been described to cause a syndromic form of X-linked nonspecific mental retardation that comprises epileptic seizures (29). Consistent with these findings, analysis of Rab GDI\(\alpha\)-deficient mice has shown that synaptic potentials remain elevated and show reduced depression during repetitive stimulation in the hippocampal CA1 region (306). In addition, the mice have increased propensity for seizures. These observations suggest that Rab GDI\(\alpha\) is essentially important for vesicle trafficking in neural cells, and its function may be needed to suppress hyperexcitability, likely through Rab3 proteins.

H. Rab Proteins and Cytoskeleton

Recently, a kinesin-like protein, termed Rabkinesin-6, has been identified as a downstream effector of Rab6, for the first time linking Rab proteins to the microtubule cytoskeleton (167). Rab6 is associated with highly dynamic tubular structures that move along microtubules from the Golgi apparatus to the cell periphery (777). Rab6 may facilitate the transport of these structures to their acceptor compartment, probably the endoplasmic reticulum, either by recruiting Rabkinesin-6 onto membranes or by changing the biochemical properties of the motor. Similarly, Rab3A and Rab3B on Ca\(^{2+}\)-dependent exocytosis is currently unknown.

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be associated with the vesicles (Fig. 7). Consistently, Rab11BP/Rabphilin-11 (432, 668, 810), a downstream effector of Rab11 mainly implicated in vesicle recycling (590, 736), directly interacts with mammalian Sec13 (433). In yeast, Sec13 counterpart is involved in Sar1-induced vesicle coat assembly (714) (see below). This result provides a physical connection between a Rab protein and a structural component necessary for vesicle budding.

VI. SAR1/ARF PROTEINS AS REGULATORS OF VESICLE BUDDING

A. Outline

The SAR1 gene was originally isolated as a multiple copy suppressor of a ts mutant of the SEC12 gene (Sar1 GEP gene) that had genetically been identified to be required for transport from the endoplasmic reticulum to the Golgi apparatus in the yeast S. cerevisiae (508). Subsequently, the SAR1 gene was also shown to be involved in transport from the endoplasmic reticulum (509, 545). There is only one SAR1 gene in yeast. Two Sar1 proteins (Sar1a and Sar1b) have been found in mammals (372).

Arf proteins are homologous to Sar1 with ~35% identity. Six Arf proteins and some Arf-like proteins (ARLs and ARD) have thus far been identified in mammals, and three members exist in S. cerevisiae (493). Arf proteins were originally purified from rabbit liver and bovine brain membranes based on their ability to stimulate in vitro the cholera toxin-catalyzed ADP-ribosylation of Gs (326, 327). The cDNAs of bovine and yeast Arf1 were then isolated (658). It was shown that Arf1 is localized to the Golgi apparatus in some cell lines and that Arf1 mutations cause secretion defects in S. cerevisiae (684). Subsequently, small G proteins involved in vesicle formation from the Golgi apparatus were identified to be Arf1 and another Arf protein (655). It is now established that Sar1 and Arf proteins are involved in the budding of vesicles from yeast to human (103, 493, 494, 634).

Mammalian Arf proteins are structurally grouped into three classes: class I including Arf1, Arf2, and Arf3; class II including Arf4 and Arf5; and class III including Arf6 (103, 494). Of these Arf proteins, Arf1 has been the most extensively characterized and is established to be involved in the budding of vesicles from the Golgi. Earlier studies implicated Arf6 in the formation of vesicles from the plasma membrane (12, 161, 162, 585, 814). However, recent studies indicate that it is also involved in remodeling of the actin cytoskeleton and cell motility downstream of Rac1 (584).

B. Coat Proteins and Vesicle Budding

Vesicle budding requires the assembly of specific proteins coating the cytoplasmic face of a donor membrane (634). The assembly of coat proteins is thought to serve at least two functions: it provides the mechanical force to pull the membrane into a bud, and it helps to capture specific membrane receptors and their bound cargo molecules. Three classes of coated vesicles have been well-characterized to date: clathrin-, COP (coat protein) I-, and COPII-coated vesicles (634, 640). Clathrin coats contain clathrin and heterotetrameric adaptor protein (AP) complexes, AP-1, AP-2, AP-3, or AP-4 (278, 350). Two different clathrin-coated vesicles containing AP-1 and AP-2 carry selected proteins from the trans-Golgi network to endosomes and lysosomes and from the cell surface to endosomes, respectively. Another AP complex, AP-3, is involved in the biogenesis of specialized organelles such as pigmented granules and synaptic vesicles and in an alternative Golgi-to-vacuolar pathway in yeast. AP-4 has recently been identified as a novel complex related to other AP complexes through searches in EST data, but its function remains to be determined. COPII-coated vesicles have been shown to be involved exclusively in the export of cargo molecules from the endoplasmic reticulum (35, 38, 634). COPI-coated vesicles seem to be involved in membrane trafficking between the endoplasmic reticulum and the Golgi apparatus, in intra-Golgi transport, and possibly in endosomes. However, it is currently a matter of debate as to how many transport steps COPI is involved in, and whether it functions in transport in the anterograde or retrograde direction or both (35, 405, 410). Sar1 and Arf proteins play crucial roles in the membrane recruitment of the COPII and COPI components, respectively (38, 405). Arf proteins are also involved in the recruitment of AP-1 and AP-3 components to the membranes (278). In contrast, neither of these small G proteins is involved in the clathrin/AP-2-coated vesicle formation from the cell surface.

C. Arf Protein Cycle: Cyclical Activation/Inactivation and Translocation

Arf proteins cycle between GDP-bound, inactive and GTP-bound, active forms, and the cycling is regulated by specific GEPs and GAPs (312, 494) (Fig. 11). This cycling is not regulated by GDIs. The GDP-bound form of Arf proteins is present in the cytosol and is converted to the GTP-bound form by the action of a GEP, inducing the conformational change that allows the myristoylated NH2-terminal amphipathic helix of the Arf proteins to interact with phospholipid bilayers. Nucleotide exchange also induces a conformational change in the switch I and II regions, allowing Arf proteins to interact with downstream effector (110, 229, 613).

Certain recent studies indicate that the myristate is exposed and can interact with the phospholipid bilayer when Arf proteins are still in the GDP-bound form (21).
This weak but measurable membrane association is completely abolished if the myristate is removed (190). Arf protein-membrane interaction permits the activation of Arf proteins by a GEP. Membrane association is stabilized in the GTP-bound form by a conformational change of the NH2-terminal helix that exposes several hydrophobic amino acid residues, including Leu-8 and Phe-9 (which are buried inside the Arf-GDP), and permits their insertion into the membrane (21, 47). A cytosolic GAP is recruited to the membrane after vesicle budding to stimulate the hydrolysis of bound GTP to GDP, resulting in the release of the Arf protein and in turn the COPI coat.

Many GEPs for Arf proteins have been isolated and characterized in yeast and mammals (312, 494, 613). Although they show different substrate specificities, they have a conserved ~200-amino acid region, called as the Sec7 domain, that shows strong similarity to yeast Sec7. Sec7 was initially identified as a protein required for transport at different stages of the yeast secretory pathway and is localized to the Golgi apparatus (195). The Sec7 domain itself was subsequently shown to be a minimum GEP unit capable of binding to and activating Arf proteins (99, 229, 440, 489, 570, 631).

Members of the Arf GEP family can be grouped into two major subfamilies on the basis of their sequence similarities and functional differences (312, 614). The high-molecular-weight Arf GEP subfamily includes yeast Sec7, Gea1, and Gea2, and mammalian BIG1/p200, BIG2, and GBF1, which all consist of 1,400–2,000 amino acid residues. Plants also have related Arf GEPs. There is a Sec7 domain in the middle of these polypeptides, as well as some additional, homologous regions. It is a notable feature of these Arf GEPs that all but one (GBF1) are sensitive to a fungal metabolite, BFA (490, 570, 631, 723), which inhibits their Arf GEP activity in an uncompetitive manner (440, 570). BIG1/p200 has been shown to be also a Golgi-localized protein in mammalian cells (440). GBF1 is colocalized with COPI to the Golgi apparatus, and, when overexpressed in cells, confers BFA resistance, suggesting that it may be involved in the formation of COPI-coated vesicles through activation of Arf proteins (122).

The second subfamily (low-molecular-weight type) includes mammalian ARNO, cytohesin-1, GRP1, and cytohesin-4 (312, 614). No homologs exist in yeast. These proteins consist of ~400 amino acids and have in common an NH2-terminal coiled-coil region, a central Sec7 domain, and a COOH-terminal PH domain. Furthermore, all are insensitive to BFA. EFA6, a GEP specific for Arf6 (191), can also be grouped into this subfamily because it is BFA insensitive and has all the structural features, despite a slightly larger size. Although earlier studies reported that ARNO, cytohesin-1, and GRP1 showed preferential GEP activity on class I and II Arf proteins, recent studies have revealed that these small GEPs are colocalized with Arf6 at the cell periphery and cause elevation of GTP-Arf6 (192, 193, 382).

ARNO prefers myristoylated Arf proteins and its activity is enhanced by PIP2 (99). In contrast, GRP1 binds to PIP2 but poorly to PIP3 through its PH domain (357). It is therefore likely that these small GEPs use the phosphoinositide-PH domain for their recruitment to a specialized membrane subdomain, where the GEPs activate myristoylated GDP-Arf proteins. Supporting this model are the recent observations that translocation of these small GEPs to the plasma membrane is stimulated by growth factors and is blocked by inhibitors of PI 3-kinases (382, 503, 751).

The crystal structure of GDP-Arf1 has revealed a patch of positively charged amino acids on its surface (16). Thus Arf1 may also interact with negatively charged phospholipids on membranes via this patch. Crystal structure analyses of 5’-guanylylimidodiphosphate [Gpp(NH)p]-bound Arf1 and of nucleotide-free Arf1 complexed with the Sec7 domain of Gea2 have provided insight into the activation mechanism of Arf1 (229). Arf1 in its inactive conformation cannot fit into the recognition surface of the Sec7 domain. Taken together with the biochemical data that a low-affinity interaction of myristoylated GDP-Arf1 with the phospholipid membrane is required for subsequent nucleotide exchange by GEPs (21, 47, 190), it is likely that phospholipids act on GEPs to induce the conformational change that permits the binding of Arf1 to the Sec7 domain, followed by Arf1 activation.
Many Arf GAPs have been identified in yeast and mammals (494, 614). These GAPs each contain a zinc fingerlike Arf GAP domain, which has been shown to be a minimum unit for the GAP activity. However, their substrate specificities have not yet been systematically characterized. An Arf GAP purified from rat spleen cytosol is activated by PIP$_2$ and shows broad substrate specificity, being active on Arf1, Arf3, Arf5, and Arf6 (153). This activity does not require Arf myristoylation. Another Arf GAP (Arf1 GAP), the first GAP identified in mammals (136), is localized to the Golgi apparatus and is stimulated by diacylglycerol, which may be produced from phosphatidylcholine by the sequential actions of phospholipase D (a downstream effector of Arf proteins; see below), and phosphatidic acid phosphohydrolase (22).

Recruitment of cytosolic Arf GAP to the vesicle membranes is enhanced by cargo receptors such as the KDEL receptor, which recognizes a Lys-Asp-Glu-Leu (KDEL) COOH-terminal motif on certain soluble proteins destined for Golgi-to-endoplasmic reticulum retrieval (23, 24). Centaurin-$\alpha$ and PIP$_2$-BP, two closely related proteins, have two PH domains that are involved in binding to PIP$_2$ (253, 710). ASAP1/DEF-1 and PAP, also close relatives, have a PH domain, three ankyrin repeats, a proline-rich region, and an SH3 domain and have been shown to interact with Src and Pyk2, respectively (19, 70, 348). Both enhance the GAPase activity of Arf1 and Arf5 but act only weakly on Arf6. PKL and GIT are closely related and contain three ankyrin repeats and a PH domain (120, 528, 731). PKL was identified as a protein that binds to the paxillin LD4 motif and serves as a connector between paxillin and molecules of the Rac1/Cdc42 pathway, PAK and PIX (731). GIT1 was identified as a protein that interacts with a G protein-coupled receptor kinase; it regulates endocytosis of various G protein-coupled receptors (120, 578). To date, the crystal structures of the GAP domain of PAP and that of Arf1 GAP complexed with Arf1 have been determined (230, 435). A unique chimera protein, ARD1, contains both Arf protein and Arf GAP structures (474). The GAP domain is specific for the Arf domain and shows GDI activity that slows the dissociation of GDP from the Arf domain (754).

D. Arf Proteins in Vesicle Budding

Although it is currently unclear at which stage Arf GEP is activated or how its activation is controlled, GTP-Arf proteins, once produced by the action of Arf GEP, associate with the Golgi apparatus and recruit coatomer, a seven subunit complex (410, 780), or a clathrin-adaptor complex, AP-1 or AP-3 (278). In the case of COPI-coated vesicle formation, GTP-Arf1 has been shown to interact with $\beta$-COP and $\gamma$-COP (780, 817, 818). However, it is currently unknown which subunits of the AP complexes interact with GTP-Arf proteins. Acidic phospholipids, such as phosphatidic acid, enhance Arf1-induced binding of coatomer (370, 614). Assembled COPI captures cargo molecules through binding to cargo receptors, such as KDEL receptor and the p24 family of proteins (410, 780). In the latter case, COPI recognizes the phenylalanine or dilyseine motif (or both) within the cytoplasmic tail of the p24 family members. Thus vesicle formation and cargo selection are coupled through a bivalent interaction of coatomer with Arf1 and a putative cargo receptor. It is speculated that coat protein assembly induces local membrane deformation into a nascent vesicle bud.

The entire process of the formation of COPI-coated and clathrin/AP-1-coated vesicles can be reproduced with pure components in vitro (677, 825). The simplest systems consist of GTP-Arf1 and either coatomer, or clathrin and AP-1, added to liposomes of defined composition that support the formation of small coated vesicles (40–70 nm diameter for COPI and 60–80 nm diameter for clathrin/AP-1) (677, 825). The results indicate that Arf1 and coatomer are sufficient to pinch off vesicles. Although it has been proposed that production of phosphatidic acid by Arf1-induced activation of phospholipase D triggers binding of coat proteins to membranes and subsequent vesicle formation, the physiological relevance of these events is unclear (612). On the other hand, a recent study has suggested that production of phosphatidic acid by acylation of lysophosphatidic acid may play a role in vesicle budding from the Golgi apparatus (773).

Either concomitant with or after vesicle budding, GTP-Arf1 is converted to the GDP-bound form by the action of a GAP. Recent studies have shown that coatomer and a transmembrane cargo receptor control membrane association of Arf1 GAP and its activity (23, 24, 230). KDEL receptor enhances the recruitment of Arf1 GAP to the membranes of the Golgi apparatus (23, 24). Arf1 GAP activity may then be stimulated by coatomer on the membranes (230). Consistent with this observation, the crystal structure of GTP-Arf1 complexed with the minimal GAP domain of Arf1 GAP indicates that, unlike most G proteins, the GAP binding site on Arf1 does not overlap with its effector binding site (230). Thus Arf1 may simultaneously interact with Arf1 GAP and coatomer, and both coatomer and cargo receptors may regulate the termination of the Arf activity.

E. Arf6 in Endocytic Recycling and Cytoskeletal Reorganization

The subcellular localization of Arf6 is quite different from that of class I and class II Arf proteins. Various studies have shown that Arf6 is involved in recycling of
endosomal vesicles and regulates receptor-mediated endocytosis (12, 161, 162, 585, 814). However, Arf6 is also implicated in remodeling of the cytoskeleton underlying the plasma membrane. Arf6 is localized to the plasma membrane, especially to membrane ruffles in spreading cells. Activation of Arf6 induces remodeling of the actin cytoskeleton and cell spreading, and expression of a dominant negative mutant of Arf6 blocks cell spreading (160, 585, 674). Furthermore, ARNO and EFA6, two low-molecular-weight Arf GEPs, have been reported to modulate growth factor- and protein kinase C-mediated cytoskeletal reorganization through activation of Arf6 (191, 193). In addition, a dominant negative mutant of ARNO inhibits both Arf6 translocation and cortical actin formation induced by growth factors (750). In this context, it is noteworthy that recent studies have suggested that there is cross-talk between the Arf6 and Rac1 pathways in actin remodeling. First, EFA6-induced cytoskeletal remodeling is blocked by coexpression of a dominant negative mutant of Arf6 or Rac1 (191). Second, a dominant negative mutant of Arf6 inhibits growth factor- and Rac1-mediated membrane ruffling (584, 813). Third, a deletion mutant of POR1/arfaptin-2, which interacts with both Rac and Arf proteins, but not a dominant negative mutant of Rac1, inhibits Arf6-mediated cytoskeletal rearrangements (160). Taken together, these results indicate that Arf6 functions either downstream of or in concert with Rac1. Further evidence suggesting a connection between Arf6 and Rac1 pathways is the finding that PKL, an Arf GAP, serves as a connector between paxillin and the Rac/Cdc42 pathway components PAK and PIX (731). An effector of Arf6 that is implicated in membrane ruffling is phosphatidylinositol 4-phosphate 5-kinase (283). This kinase translocates to ruffling membranes and produces PIP₂ synergistically with Arf6 and phosphatidic acid, the production of which is catalyzed by phospholipase D. Because phospholipase D itself is an effector of Arf proteins and PIP₂ is an activator of Arf proteins, it is tempting to speculate that the local phospholipid metabolism that is regulated by Arf6 may play a crucial role in membrane ruffle formation.

F. Sar1 as a Regulator of Vesicle Budding

The function of Sar1 in vesicle budding has been extensively characterized in the yeast S. cerevisiae but in less detail in mammals (38, 634). Sar1 does not undergo any known lipid modification, but it is associated with the endoplasmic reticulum and is involved in the formation of COPII-coated transport vesicles from the endoplasmic reticulum (39, 518, 544). The Sar1 cycle is regulated by a GEP (Sec12) and a GAP (Sec23) (40, 44, 371, 808). Sec12 is an integral endoplasmic reticulum membrane protein involved in activation of Sar1 (40, 142, 143). However, its mammalian homolog has not yet been identified. Sec23 accelerates the GTPase activity of Sar1 and is tightly associated with Sec24 (the Sec23 complex) (808). Sec23 homologs are present in mammalian cells, and they localize to compartments between the endoplasmic reticulum and the Golgi apparatus (554, 558). A Sec24 homolog is also present in mammalian cells, and as expected, it interacts with mammalian Sec23 (559, 715). Thus the Sar1/COPII system is well conserved between yeast and mammalian cells.

COPII assembly and disassembly are regulated by the Sar1 cycle (44, 371, 679). Sec12-induced activation of Sar1 promotes association of the Sec23 complex to the budding site at the endoplasmic reticulum. Then, the Sec13 complex comprised of Sec13 and Sec31 is recruited onto the Sec23 complex, and this binding and the subsequent polymerization of the Sec13 complex are proposed to lead to the concentration of cargo proteins and the deformation of the membrane into a coated bud. Thus vesicles coated with GTP-Sar1, the Sec23 complex, and the Sec13 complex, are produced. Sec23-induced GTP hydrolysis of Sar1 leads to dissociation of these proteins from the vesicle, although hydrolysis appears not to be a prerequisite for the vesicle budding. Two other factors have been implicated in COPII-vesicle budding. Sec16 interacts directly with the Sec23 complex and may serve as a scaffold for COPII coat assembly (174, 225). Sed4 is highly homologous to Sec12 in its cytoplasmic domain, although it does not seem to show GEP activity on Sar1. However, unlike Sec12, Sed4 interacts directly with Sec16 (224). It is thus possible that Sar1, along with Sed4 and Sec16, may be involved in a distinct vesicle formation process.

Proteins necessary for the targeting and docking processes, such as v-SNARE and Rab proteins, must be incorporated into transport vesicles. However, the mechanism for this is currently unknown. Two facts are established. 1) The Sec23 complex interacts simultaneously with GTP-Sar1 and two v-SNAREs, Bet1 and Bos1, in a cooperative manner during the formation of endoplasmic reticulum-to-Golgi COPII transport vesicles (678), and 2) that Rab11BP/Rabphilin-11 (432, 668, 810), a downstream effector of Rab11 mainly implicated in vesicle recycling (590, 736), interacts directly with mammalian Sec13 (433).

VII. RAN FUNCTION IN NUCLEOCYTOPLASMIC TRANSPORT AND MICROTUBULE ORGANIZATION

A. Outline

There is only one Ran gene in many cell types (including human and Schizosaccharomyces pombe), although cells of other species (S. cerevisiae and tomato)
contain two or more closely related Ran genes (483). Ran protein (Ras-related nuclear protein: Ran) was originally cloned on the basis of its homology to Ras proteins (158). The first evidence for the involvement of Ran in nuclear transport was obtained in 1993 by showing that Ran is essential for the nuclear import in permeabilized cells of a reporter construct containing the nuclear localization signal (NLS) of the simian virus 40 T antigen (PKKKRKV) (464, 484). It is now clear that Ran plays a central role in nucleocytoplasmic transport. Recent studies have uncovered another role for Ran in microtubule organization during the M phase of the cell cycle (85, 325, 329, 351, 504, 533, 781).

B. Nucleocytoplasmic Transport

Macromolecules are transported back and forth between the cytoplasm and the nucleus through NPCs. The NPCs contain more than 50 different proteins (454, 517, 536, 565). Movement of macromolecules through the NPCs occurs by at least two distinct mechanisms: passive diffusion and active transport (180). Small molecules diffuse quickly through the NPCs in either direction, whereas those larger than 50–60 kDa, including proteins and RNAs, are actively and selectively transported. The transport of cargo proteins requires at least three types of soluble factors: transport receptor molecules, adaptor molecules, and Ran and its binding proteins (139, 234, 454). Transport receptors shuttle continuously between the nucleus and the cytoplasm, interact with NPCs, bind cargo molecules, and facilitate cargo translocation through the NPCs. There are two types of transport receptors: nuclear import receptors, called importins, and export receptors, called exportins (734). Importins and exportins recognize NLS and nuclear export signal (NES) of cargos, respectively, bind them, and transport bound cargos through the NPCs (3, 483). In some cases, transport receptors do not directly bind cargos and indirectly bind them through adaptors that can recognize their NLS. For instance, importin-α and importin-β are well-known adaptor and receptor molecules, respectively. Transport receptors constitute a superfamily of proteins that share a binding domain of GTP-Ran (189, 235). In the yeast S. cerevisiae, 14 members of importin-β superfamily are predicted from the genome sequence (3). Fewer receptors have been identified in mammals, but more receptors will be found in the future.

C. Ran Cycle: Cyclical Activation/Inactivation and Translocation

Like other small G proteins, Ran is cyclically activated and inactivated, but the most characteristic feature of the Ran cycle is that the GTP-bound form and the GDP-bound form are asymmetrically distributed in the nucleus and the cytoplasm, respectively. This asymmetric distribution of the two forms are due to asymmetric distributions of the regulators, a GEP and a GAP (Fig. 12). Only one Ran GEP, regulator of chromosome condensation (RCC1) (54, 538), and only one Ran GAP, Ran GAP1 (51, 52), have been identified in mammals. RCC1 was originally identified as a regulator of chromatin condensation (538). RCC1 is localized exclusively in the nucleus where it is associated with the chromatin (539) and converts the GDP-bound form to the GTP-bound form (54). In contrast to RCC1, Ran GAP1 is localized exclusively in the,

![Figure 12. Cyclical activation/inactivation of Ran and its translocation.](http://physrev.physiology.org/Downloadedfromhttp://physrev.physiology.org/)
cytoplasm (455, 596) but some can be detected in the nucleus (726). In vertebrate cells, a significant portion of Ran GAP1 acquires a 121-amino acid ubiquitin-like addition (called SUMO-1) (430, 455). This modification does not target this protein for degradation but instead targets it specifically to the filaments at the cytoplasmic face of the NPCs. Moreover, two GTP-Ran-binding proteins, Ran-binding protein 1 (RanBP1) and Ran-binding protein 2 (RanBP2/NUP380), have been isolated (53, 784, 805). RanBP1 is localized in the cytoplasm and enhances the Ran GAP1 activity (53, 127, 597, 637), whereas RanBP2 is a component of the NPCs at the cytoplasmic face that recruits SUMO-Ran GAP1 and enhances its GAP activity (430, 455). Once GTP-Ran is produced in the nucleus, an exportin binds both GTP-Ran and its cargo in a cooperative manner, and this ternary complex is exported to the cytoplasm through the NPCs. At the cytoplasmic face of the NPCs, GTP-Ran in this complex is attacked by SUMO-Ran GAP1 and Ran BP2 (430, 455, 622), and in the cytoplasm it is attacked by Ran GAP1 and RanBP1, leading to conversion to GDP-Ran (50, 186, 409). Inactivation of GTP-Ran induces the dissociation of the complex in the cytoplasm. GDP-Ran then forms a complex with another regulator, named p10/NTF2, which transports GDP-Ran back to the nucleus through the NPCs (595). p10/NTF2 has a GDI activity, keeping Ran in the GDP-bound form (789). GDP-Ran thus returned to the nucleus and is then reutilized for the next cycle of transport. Thus the Ran cycle is regulated by many factors. The most important aspect of this cycle is that GTP-Ran forms a gradient across the nuclear envelope and that this gradient plays a key role in controlling the directionality of nucleocytoplasmic transport (236, 311). Ran does not undergo any post-translational lipid modification and probably, as a result, does not bind to membranes inside the cell or require lipids for its activity (618).

D. Mode of Action of Ran in Nucleocytoplasmic Transport

As described above, one role of Ran is to export cargos via exportins from the nucleus to the cytoplasm. The most well-characterized exportin is CRM1 (also called exportin 1), which recognizes a specific, leucine-rich type of NES (188, 203, 682). In the presence of GTP-Ran, both GTP-Ran and a cargo bind to CRM1 in a cooperative manner to form a GTP-Ran-CRM1-cargo complex (Fig. 13A). This complex is exported to the cytoplasm, where GTP-Ran is converted to GDP-Ran, causing the cargo to dissociate from CRM1. CRM1 is then reimported to the nucleus in the empty state. In this example, CRM1 functions without an adaptor. In some cases, NES-containing proteins bind RNAs that are destined for nuclear export. In these cases, the NES proteins serve as adaptors and the cargo is the RNA.

Ran has another role in nuclear import (536). In the absence of GTP-Ran, importin-β forms a complex with

![Fig. 13. Mode of action of Ran in nucleocytoplasmic transport. A: mode of action of Ran in nuclear export. B: mode of action of Ran in nuclear import.](http://physrev.physiology.org/10.1152/jn.00579.2012)
importin-α that serves as an adaptor, binding NLS-containing proteins (Fig. 13B). This complex is imported into the nucleus through the NPCs, where it encounters GTP-Ran. GTP-Ran binds to importin-β, causing dissociation of the complex into each component. Empty importin-β, probably still bound to GTP-Ran, is then reexported to the cytoplasm. Recycling of importin-α requires a specific exportin, named CAS, that is analogous to CRM1 (377). The binding of importin-α to CAS requires cooperative GTP-Ran binding, and CAS prefers importin-α, which is not associated with a cargo. Thus empty importin-α is exported again by CAS. After inactivation of GTP-Ran to GDP-Ran in the cytoplasm, importin-α dissociates, and CAS returns to the nucleus for reutilization.

During nuclear transport, the nucleotide-bound state of Ran acts as a simple switch to delineate the direction of movement, and the energy of GTP hydrolysis is not strictly required. Moreover, the binding of GTP-Ran to importins induces conformational changes so that they dissociate cargos. GTP-Ran is not essential for the transportation of importins through the NPCs, because the NPC-interacting region of importins alone can be transported through the NPCs in the absence of GTP-Ran (3).

E. A Role for Ran in Microtubule Organization

During G1, S, and G2 phases of the cell cycle, nucleocytoplasmic transport is active, but during the M phase, the nuclear envelope is broken down and nucleocytoplasmic transport stops. At the onset of mitosis in eukaryotes, the nuclear envelope and interphase microtubule array disassemble, and the duplicated centrosomes nucleate microtubules that interact with chromosomes, forming a bipolar spindle. Sister chromosomes balanced at the spindle equator segregate and move to opposite spindle poles during anaphase. How chromosomes influence spindle assembly in the absence of microtubule-nucleating organelles has been a long-standing mystery. Recent studies have revealed that Ran and its novel effector, named Ran-binding molecule (RanBPM) (504), regulate aster formation and spindle assembly.

An initial hint for Ran involvement in microtubule regulation was derived from the observation that, in the yeast S. cerevisiae, overexpression of Prp20, the yeast RCC1 homolog, suppresses the toxic effect of certain hyperstable α-tubulin mutants (351). More direct evidence has recently been obtained by the identification of a novel mammalian GTP-Ran-binding protein, RanBPM (504). RanBPM associates with centrosomes, the microtubule-nucleating centers of mammalian cells. Overexpression of RanBPM in cultured cells or addition of GTP-Ran or GTPyS-Ran to a cell extract induces aster formation. Conversely, inhibition of RanBPM or Ran activity prevents in vitro aster formation (85, 329, 533, 781). The precise mode of action of Ran and RanBPM in spindle formation is not understood, but in the absence of a nucleus, chromatin-bound RCC1 and cytoplasmic Ran GAP1 produce a natural gradient of GTP-Ran that may be most concentrated at the chromosome surface (325). Consequently, the GTP-Ran-RanBPM complex would stimulate microtubule assembly adjacent to the chromosomes. In this case, GTP hydrolysis is not essential for microtubule assembly but may have some secondary role in the elongation of previously nucleated microtubules. Other cellular factors, including the chromosome-associated, kinesin-like protein, XKLP1 (752, 764), and a complex of cytoplasmic dynein, dynactin and NuMA (466, 801), are also involved in organizing these microtubules into spindles. Thus the observation that Ran and RanBPM stimulate microtubule polymerization offers a significant insight into the process by which chromosomes drive spindle assembly.

VIII. SMALL G PROTEIN CASCADES AND CROSS-TALKS

A. Small G Protein Cascades

Multiple small G proteins form a signal cascade and thereby transduce their signals to downstream effectors. For instance, in the yeast S. cerevisiae, Rsr1, a member of the Ras family, is thought to be first activated by an unknown cue that is produced by the previous budding site (45, 96) (Fig. 14). In the next step, GTP-Rsr1 binds to Cdc24, a GEP for Cdc42, which in turn binds GDP-Cdc42 to activate it (821). The activation of Cdc42 induces not only reorganization of the actin cytoskeleton but also the recruitment of multiple small G proteins, including Rho1 and Sec4, to the future budding site. Sec4 would supply the bud with vesicles necessary for bud enlargement (527, 623), while Rho1 would induce synthesis of the new cell wall component, 1,3-β-glucan, by directly activating 1,3-β-glucan synthase and stimulating expression of the genes necessary for this synthesis (80, 157, 456, 580). Rho1 would also regulate reorganization of the actin cytoskeleton necessary for the budding processes (200, 304, 363, 739).

Another typical example of small G protein cascade includes Rho/Rac/Cdc42 proteins in mammalian cells (420, 744) (Fig. 15). Rho proteins regulate formation of stress fibers and focal adhesions (564, 600, 601), Rac proteins regulate formation of lamellipodia and membrane ruffles (521, 522, 602), and Cdc42 proteins regulate formation of filopodia (368, 522). The sequential activation of these three small G proteins by extracellular agonists has been best characterized in quiescent Swiss 3T3 fibroblasts (522, 600, 602). Agonists like bradykinin activate Cdc42 proteins in these cells to produce filopodia or...
microspikes. Activation of Cdc42 proteins leads to localized activation of Rac proteins; hence, filopodia are often associated with lamellipodia, which are produced by Rac proteins. In fact, it is hard to see filopodium formation unless Rac activity is first inhibited. Quiescent Swiss 3T3 cells have no detectable stress fibers, and activation of Rac proteins under these conditions leads to weak and delayed activation of Rho proteins that produces stress fibers. This typical cascade of Rho/Rac/Cdc42 proteins observed in Swiss 3T3 fibroblasts has not always been seen in other cell lines. For instance, it has been shown that Rho proteins are inhibited by Rac/Cdc42 proteins in cultured cells such as neuroblastoma cells, N1E-115 cells, and NIH 3T3 cells (277, 388). In addition, Rho proteins may in turn inhibit the activity of Rac/Cdc42 proteins in N1E-115 cells (624).

B. Cross-talk Between Small G Proteins

In addition to the sequential cascade of multiple small G proteins, distinct families of small G proteins regulate various cellular functions in a cooperative manner. Although a dominant active mutant of Rho proteins does not cause transformation of fibroblasts, dominant negative mutants of Rho proteins inhibit Ras protein-induced transformation (341, 583). Similarly, a dominant negative mutant of Rac/Cdc42 proteins inhibits Ras protein-induced transformation, while a dominant active mutant of these Rac/Cdc42 proteins enhances oncogenic Ras protein-triggered, morphologic transformation (581, 582). Thus Rho/Rac/Cdc42 proteins are involved in a cooperative manner in Ras protein-induced transformation.

It has recently been shown that Rho/Rab proteins coordinately regulate cell adhesion and migration of cultured MDCK cells (303). TPA induces cell scattering of MDCK cells and induces early disassembly of stress fibers and focal adhesions followed by their reassembly in MDCK cells. Expression of a dominant active mutant of RhoA inhibits the TPA-induced disassembly of stress fibers and focal adhesions, while microinjection of C3 blocks their reassembly. In addition, microinjection of C3 or a dominant active mutant of RhoA inhibits the TPA-
induced cell scattering. In contrast, expression of Rab GDI or a dominant negative mutant of Rab5 attenuates either the TPA-induced reassembly of stress fibers and focal adhesions and subsequent cell scattering of MDCK cells (303). Expression of a dominant active mutant of Rac1 or Cdc42 inhibits the HGF/SF- or TPA-induced cell scattering (331, 361). Therefore, during cell migration at least, Rho/Rac/Cdc42 proteins may regulate the reorganization of the actin cytoskeleton, while Rab5 may control the recycling of adhesion molecules such as integrins by intracellular vesicle trafficking. The temporal and spatial activation and inactivation of these two families of small G proteins appear to be required for reorganization of the actin cytoskeleton and cell migration.

IX. CONCLUSIONS AND PERSPECTIVES

A. Roles in Two Types of Cell Regulation

The mechanism of \( G_s \)-induced activation of adenylate cyclase is different from that of EF-Tu-induced elongation of polypeptide. Once GTP-\( G_s \) binds to adenylate cyclase, it continuously activates it until it is converted to the GDP-bound form. In contrast, GTP-EF-Tu elongates a single amino acid, and further elongation requires the inactivation and reactivation of EF-Tu (60) (Fig. 16). GTP-\( \gamma \)-S-G, continuously activates adenylate cyclase, whereas GTP-\( \gamma \)-S-EF-Tu stimulates just one cycle of peptide elongation and stops further elongation. In this sense, Ras-induced Raf protein kinase activation and Rho/Rac/Cdc42 protein-induced activation of their specific downstream effector protein kinases are similar to the \( G_s \)-induced adenylate cyclase activation, whereas reorganization of the actin cytoskeleton regulated by Rho/Rac/Cdc42 proteins, vesicle trafficking regulated by Rab and Sar1/Arf proteins, and nucleocytoplasmic transport regulated by Ran are similar to the EF-Tu-induced elongation of polypeptide and require cyclical activation and inactivation of the small G proteins. Consistent with this mode of action of \( G_s \) and Ras proteins, the ADP-riboylation of \( G_s \) by cholera toxin continuously activates adenylate cyclase, thereby causing diarrhea (733), and point-mutated activated forms of Ras proteins frequently found in a variety of cancers continuously activate Raf protein kinases that cause stimulated cell proliferation (82). In contrast, no point-mutated activated forms of other small G proteins have been identified, and this may be due to the necessity for the cyclical activation and inactivation of these small G proteins for their cell functions. Instead, many GEPs for Rho/Rac/Cdc42 proteins have been identified as oncogenes (93, 251, 744). Oncogenic GEPs increase relative ratios of small G protein GTP-bound forms to GDP-bound forms and show oncogenic phenotypes. From this point of view, we should carefully interpret data obtained using dominant active mutants of small G proteins.

B. A Role as Biotimers Rather Than as Molecular Switches

All downstream effectors of small G proteins have at least two functionally distinct domains: a small G protein-
binding domain and a catalytic domain (in the case of enzymes) or a protein-interacting domain (in the case of nonenzymes). In the case of enzymes, the binding of the small G protein induces conformational change, resulting in its activation or inactivation. The activation (or inactivation) continues until the small G protein is converted to the GDP-bound form by the action of GAPs, causing its release from the enzyme. In the case of nonenzymes, the binding of the small G protein induces conformational change in the effector, resulting in the interaction with or the dissociation from a further downstream effector. The interaction or the dissociation continues until the small G protein is converted to the GDP-bound form. In both cases, the small G protein-binding region directly or indirectly masks the catalytic domain or the other protein-binding domain, and the binding of the small G protein induces unfolding of this functional domain. In this sense, small G proteins work as more than molecular switches, that activate or inactivate the downstream effectors, and work as biological timers (biotimers), that induce activation or inactivation and determine the periods of the functioning time (Fig. 17). It would be, therefore, of crucial importance to understand the mechanisms when and how small G proteins are activated by GEPs and inactivated by GAPs.

### C. A Role as Spatial Determinants

In addition to temporal regulation of small G proteins, spatial regulation is also essential for them to regulate various cell functions, particularly very dynamic cell functions, such as cell migration and adhesion. The sites of the activation of some small G proteins, such as Ras, Sar1, and Ran proteins, are determined by their GEPs. Upon stimulation of membrane receptors, Ras GEPs, such as SOS, are recruited to the cytoplasmic region of the plasma membrane receptors through adaptors, such as GRB2/SHC, where Ras proteins are activated (75, 399, 549, 617). A Sar1 GEP, Sec12, is a transmembrane protein of the endoplasmic reticulum and interacts with GDP-Sar1 and activates it at the cytoplasmic surface of the endoplasmic reticulum (40, 142, 143). A Ran GEP, RCC1, is associated with chromatin and activates it in the nucleus (54, 539). In these cases, however, it remains unknown how activities of these GEPs are regulated. Chromatin structure may regulate RCC1 activity and cargo proteins may regulate Sec12 activity. There is another possibility that they are always active without being regulated and attack their substrates when they are available. An Arf1 GEP, ARNO, binds to PIP2 and/or PIP3 and is associated with a specialized membrane subdomain, where it interacts with GDP-Arf1 and converts it to the GTP-bound form (99). Thus lipid metabolism is crucial for the localization of Arf1.

In contrast to these small G proteins, Rab proteins stay in the cytosol in the GDP-bound form complexed with Rab GDIs and they are translocated to their specific membrane compartments where they are activated (572, 701–703). GEPs may also be determinants for their localization, but Rab3 GEP is mostly found in the cytosol, and there is no evidence that Rab3 GEP is first associated with membranes and then recruits Rab3 proteins (543, 762). Another possible mechanism is that the downstream effector(s) may serve as determinants. A downstream effector of Rab3 proteins, Rabphilin-3, is associated with synaptic vesicles in a Rab3A-independent manner, and upon its activation, it is associated with the vesicles through Rabphilin-3 (460, 654, 664). However, there is a contradictory result that the association of Rabphilin-3 with synaptic vesicles is dependent on GTP-Rab3A (397, 683). Rab5 proteins are also reported to be first associated with endosomes, followed by recruitment of their downstream effector, Rabaptin-5, and the COOH-terminal regions of Rab5 proteins are essential for this specific localization (685). These mechanisms are similar to the Ras protein system in which the Ras proteins activated at the cytoplasmic surface of the plasma membrane recruit their downstream effector, Raf protein kinases (389, 687). However, it is unknown how these activated Rab proteins determine their specific localizations. They could be physically associated with membrane phospholipid through their COOH-terminal prenyl moieties (27, 322, 340, 349, 568, 765), but lipid-lipid interactions alone presumably do not determine their specific localizations. It also remains unknown how these GEP activities are regulated. The situation is similar for Rho/Rac/Cdc42 proteins. Most Rho/Rac/Cdc42 proteins stay in the cytosol in the GDP-bound form complexed with Rho GDIs (702, 704). Upon activation by their specific GEPs, they are translocated to their functioning sites. Some GEPs, such as Tiam1 for Rac proteins and frabin for Cdc42, require specific localizations through the membrane or F-actin-binding domains for their physiological functions (469, 539).
When dominant active mutants of Rac or Cdc42 proteins are overexpressed, they could be artificially distributed in the cell, and some of them are located at the real functioning site and show similar effects there. Under physiological conditions, small amounts of Rac or Cdc42 proteins activated by their GEPs at specific sites are involved in their functions. In many literatures, overexpressed dominant active mutants of these proteins show a variety of phenotypes, but we should be careful for their interpretations. It is of crucial importance to understand the mechanisms of the temporal and spatial activation and inactivation of these small G proteins.

We thank Drs. Yoshito Kajiro, Kenichi Kariya, Tohru Kataoka, Akira Kikuchi, Akiko Nakano, Kazuhisa Nakayama, Takeharu Nishimoto, Fuyuhiko Tamanoi, Kazuma Tanaka, and Yoshihiro Yoneda for their valuable comments on the manuscript.

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