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ROLE OF SMALL G PROTEINS IN YEAST CELL POLARIZATION AND WALL BIOSYNTHESIS[1]

Enrico Cabib, Jana Drgonová, and Tomás Drgon
National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; e-mail: enricoc@bdg10.niddk.nih.gov, janad@bdg10.niddk.nih.gov, tomasd@bdg10.niddk.nih.gov

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ABSTRACT

In the vegetative (mitotic) cycle and during sexual conjugation, yeast cells display polarized growth, giving rise to a bud or to a mating projection, respectively. In both cases one can distinguish three steps in these processes: choice of a growth site, organization of the growth site, and actual growth and morphogenesis. In all three steps, small GTP-binding proteins (G proteins) and their regulators play essential signaling functions. For the choice of a bud site, Bud1, a small G protein, Bud2, a negative regulator of Bud1, and Bud5, an activator, are all required. If any of them is defective, the cell loses its ability to select a proper bud position and buds randomly. In the organization of the bud site or of the site in which a mating projection appears, Cdc42, its activator Cdc24, and its negative regulators play a fundamental role. In the absence of Cdc42 or Cdc24, the actin cytoskeleton does not become organized and budding does not take place. Finally, another small G protein, Rho1, is required for activity of $\beta(1 \rightarrow 3)$glucan synthase, the enzyme that catalyzes the synthesis of the major structural component of the yeast cell wall. In all of the above processes, G proteins can work as molecular switches because of their ability to shift between an active GTP-bound state and an inactive GDP-bound state.

CONTENTS

INTRODUCTION

During both vegetative proliferation and sexual conjugation, the yeast cell undergoes localized morphogenetic changes that are preceded by cell polarization. Polarization and morphogenesis occur at specific stages of these processes and thus require temporal and spatial regulation. How does the cell dictate the time and place for new morphological changes to occur? Evidence accumulated in the past decade indicates that the timing and localization involve the concerted interactions of a large number of molecules. Most prominent among the regulatory factors that control these processes are small GTP-binding proteins of the Ras superfamily. Small G proteins are especially suited to function as molecular switches because of their ability to shift between a GTP-bound active form and a GDP-bound inactive form. These changes are regulated by other proteins: GTPase-activating proteins (GAPs), which enhance the intrinsic GTPase activity of the G protein, thus stimulating the transition from GTP- to GDP-bound state; GTP-GDP exchange factors (GEFs), which at high GTP-GDP ratios found in the cell lead to an increase in the GTP-bound form of the protein; and GDP dissociation inhibitors (GDIs). In the small G protein systems, GAPs function as negative regulators and GEFs as activators, whereas GDIs tend to keep the G protein in the cytoplasm in an inactive state (1). The participation of small G proteins in cell polarization and in subsequent morphogenesis is the subject of this review.

In both the formation of a new bud and the construction of the pointed projection that precedes sexual conjugation, three consecutive steps can be recognized: (*a*) choice of a site at the cell cortex where the new growth will occur;

(*b*) organization of the site, including assembly of the machinery required for subsequent growth; (*c*) actual building of the new structure, the morphogenetic step. These three stages are considered below. Lack of space precludes discussion of pseudohyphal growth, although small G proteins have recently been implicated in regulation of this morphogenetic process (2).

WHERE TO START

Yeast cells, which are usually ovoid, develop polarity and display polarized growth in two different modes of their life cycle: budding and mating. In the vegetative cycle, yeast cells divide by budding, and the position of bud emergence is predetermined. *Saccharomyces cerevisiae* cells have long been known to exhibit two different budding patterns (3–6), depending on their ploidy. Haploid yeast cells exist in two mating types: **a** and α. Both **a** and α cells exhibit so-called axial budding, in which a new bud always emerges at the cell pole where budding occurred previously. Diploid cells, on the other hand, display so-called polar budding. In this case, a new bud emerges at either of the cell poles (Figure 1).

Cells of each mating type secrete a distinct mating pheromone, and at the same time they sense the pheromone of their mating partner. When cells of the two mating types come together, they stop dividing (arrest) and develop a polarized growth projection toward their mating partner. Eventually, cells fuse at the tips of these projections, giving rise to a diploid zygote.

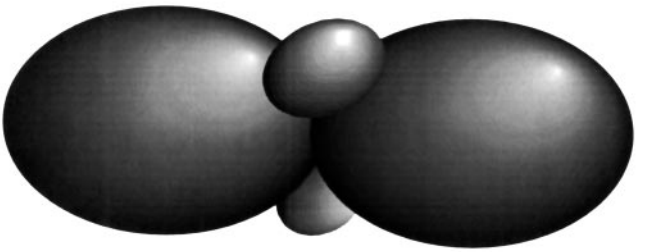
In recent years, much has been learned through genetic studies about the mechanisms underlying the budding pattern (7–10a, 11). Genes isolated with a variety of screens were systematized, and four gene classes were identified with respect to bud site selection:

1. Genes that are responsible for establishment of nonrandom (either axial or polar) budding patterns [*BUD1* (12), *BUD2* (13–15), and *BUD5* (16, 17); for a list of most of the genes mentioned in this review, see Table 1]. Mutants in these genes exhibit a random budding pattern but do not show any growth impairment. Genes in this group are required for proper function of axial and polar genes in classes 2 and 3 (12, 13, 17–19).

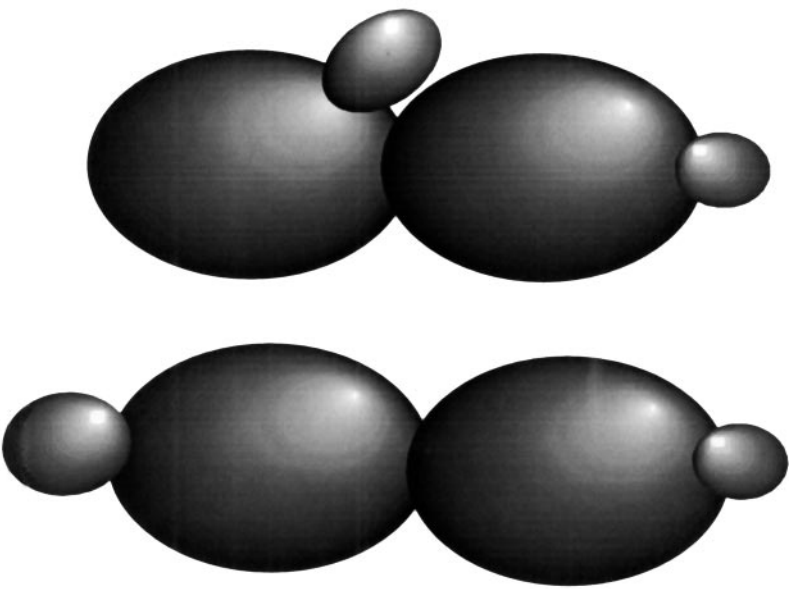
2. Genes that are responsible for development of the axial budding pattern displayed by **a** or α haploid cells; i.e. *BUD3*, *BUD4* (12, 20, 21), *AXL1* (22), *AXL2* /*BUD10* (23, 24). Mutants in these genes, despite being haploid, exhibit a polar budding pattern without any significant growth defect.

3. Genes required for the polar budding pattern in diploids [*ACT1* (25, 28), *SPA2* (26), *RVS161*, *RVS167* (27, 29), *BNI1*, *BUD6*, *BUD7*, *BUD8*, *BUD9*

haploid cells
axial budding



diploid cells
bipolar budding



(30, 31)]. Diploids mutated in these genes bud randomly or manifest a bias for one of the poles in bud site selection. The budding pattern of haploid cells is not affected by mutations in these genes.

4. Genes required for organization of the bud site. Mutants in these genes are unable to bud; they are discussed in a later section.

The molecular principles underlying development of cell polarity are conserved among many eukaryotic organisms, including mammals and plants. Yeast proteins involved in these processes have close homologues in other eukaryotic cells (32–35, 35a, and references therein).

Bud Site Selection

Chronologically, the first step in polarity development in yeast is to mark the site where growth will occur (Figure 2). That a physical landmark exists at this site is supported by the finding that in haploid cells each succeeding bud site is immediately adjacent to the preceding one, as determined from the distribution of bud scars remaining after cell division (36). In addition to septin proteins (discussed later in this section), the landmark may consist of Bud3 (20), Bud4 (21), and Axl2/Bud10 (23, 24). These proteins form a ring around the mother-bud neck that splits during cytokinesis and is retained on both the mother and daughter cells (8, 20, 21, 23, 24). In diploid cells, preferred polar bud sites exist, but their nature is not well understood (12). Candidate genes that may participate in polar bud site marking in diploid cells (*ACT1*, *SPA2*, *BNI1*, *BUD6*, *BUD7*, *BUD8*, *BUD9*) are discussed in reference 19.

The next step, recruiting the budding machinery to the site delimited by the landmark, is common to both axial and polar budding. Execution of this step is dependent on a small G protein, Bud1, and its regulators, Bud2 and Bud5 (12, 13, 17, 18). If any of these proteins is defective or absent, cells establish a bud site at random (Figure 2; 13, 16–18). Nevertheless, even if the bud site is chosen randomly, it is functional and indistinguishable from a nonrandomly selected one.

Site selection is followed by accumulation of various structures at the selected site, one of which is a microfilament ring composed of at least four proteins now called septins (37): Cdc3 (38), Cdc10 (39), Cdc11 (40), Cdc12 (39). Septins are often referred to as GTP-binding proteins on the basis of protein sequence analysis. However, no data showing actual GTPase activity or

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Figure 1 Schematic representation of different budding patterns in yeast. Haploid cells bud axially, the new bud emerging always adjacent to the site of previous budding. Diploid cells bud polarly, the new bud emerging at either of the cell poles.

Table 1 Genes involved in bud site selection and organization and in cell wall synthesis

Gene	Proposed function of encoded protein	References
ACT1	Actin; cytoskeleton, bipolar bud site selection	25, 28
AXL1	Similar to insulin-degrading enzymes; axial bud site selection	22
AXL2/BUD10	Axial bud site selection	23, 24
BEE1/LAS17	Homologous to Wiskott-Aldrich syndrome protein; nucleation of actin	66, 67
BEM1	SH3 domains; cell polarization	54
BEM2	GTPase-activating protein (GAP) for Rho1	69, 135
BEM3	GAP for Cdc42	71
BEM4	Chaperone for small G proteins	100
BNI1, *BNR1*	Formins; organization of actin cytoskeleton	73–75, 102
BOI1, *BOI2*	Interact with Bem1	101
BUD1/RSR1	Small Ras-like G protein; general bud site selection	12, 18
BUD2	GAP for Bud1	13–15
BUD3, *BUD4*	Axial landmark proteins; axial budding	12, 20, 21
BUD5	GTP-GDP exchange factor (GEF) for Bud1	16, 17
BUD6, *BUD7*, *BUD8*, *BUD9*	Bipolar budding	30, 31
CDC3, *CDC10*, *CDC11*, *CDC12*	Septins; components of the neck filament ring	37–40
CDC24	GEF for Cdc42	71
CDC42	Rho-like small G protein; bud site organization, pheromone signaling	49, 65, 88, 89
CDC43	Geranylgeranyl transferase subunit; prenylation of Cdc42	92, 93
CLA4	Protein kinase; homologous to Ste20, cell morphogenesis	84
FKS1, *FKS2*	$\beta(1 \rightarrow 3)$glucan synthase subunits	124–126
LRG1	GAP for Rho proteins	137
PCA1	Nucleation of actin	66
PKC1	Protein kinase C; triggers MAP kinase cascade involved in maintenance of cell wall integrity	139–144
RGA1/DBM1	GAP for Cdc42	95, 136
RHO1	Small G protein; activator of $\beta(1 \rightarrow 3)$glucan synthase and protein kinase C; actin organization	74, 122, 123, 139, 140
RHO2	RHO1 homolog of uncertain function	120
RHO3, *RHO4*	Small G proteins; actin organization, polarity maintenance?	121, 150–152
ROM1, *ROM2*	GEF for Rho1	134
RVS161, *RVS167*	Bipolar budding	27, 29
SAC7	GAP for Rho1	98
SPA2	Bipolar budding	26
STE20	Protein kinase; pheromone signaling	83, 86, 90
TOR2	Phosphatidylinositol kinase; actin organization	97

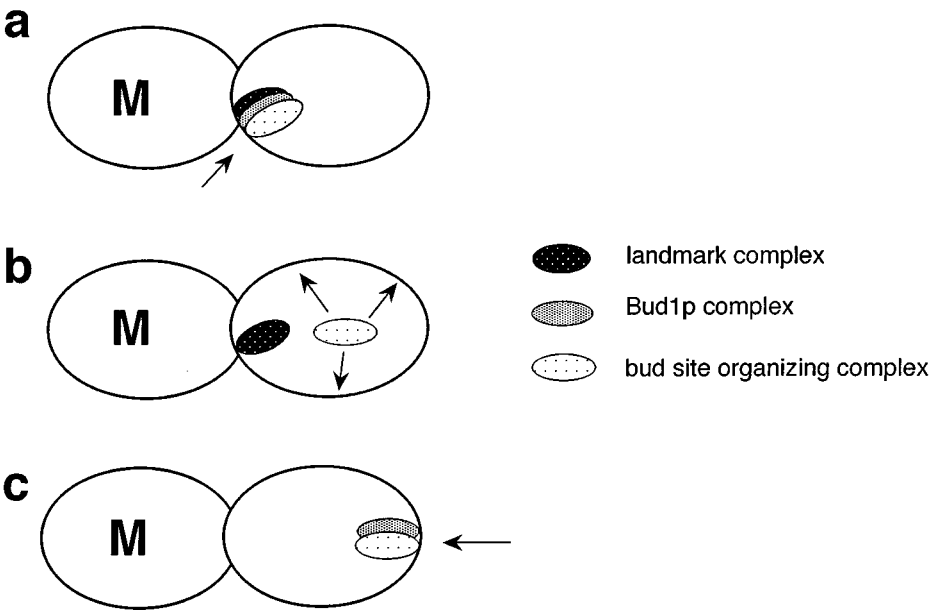

Figure 2 Bud site selection and construction. (*a*) The participation of different protein complexes in bud site selection may be rationalized by assuming that Bud1, together with Bud2 and Bud5, acts as a mediator between the proteins involved in establishing a landmark (Bud3, Bud4, Axl1, and Axl2) and those that organize the bud site. (*b*) In the absence of Bud1, the bud site organizers are not able to recognize any landmark, and budding occurs randomly. (*c*) If the landmark is defective, Bud1 and associated proteins are still able to guide the organizers to a default site and polar budding occurs. *Arrows* point to positions where a new bud will emerge. *M*, mother cell.

GTP binding are available. Analysis of mutants showed that the microfilament ring is necessary for normal growth of the bud; however, septins may also be involved in establishment of the landmark that delineates the site for budding in the next division. Thus, some temperature-sensitive mutants in septin genes, when grown as haploids at a restrictive temperature, do not form buds at their normal axial location (20, 25, 41). Moreover, in a *cdc12^{ts}* mutant, the typical localization of Bud3 (20, 38), Bud4 (21), and Bud10/Axl2 (23, 24) in a double ring at the bud neck is lost or disturbed. Bud10/Axl2, however, still localizes to nascent and small buds in this mutant. Therefore, it is possible that in axially budding cells, septins serve as an anchoring site for other proteins or structures, such as Bud3, Bud4, and Bud10/Axl2.

In this review, we focus on those components of the bud site selection machinery that have been shown to operate on the basis of GTP-GDP cycling.

Components of the Bud Site Selection Apparatus

RSR1/BUD1 was isolated as a multicopy suppressor of a *cdc24* mutation that causes inability to establish cell polarity and to bud (18). Sequence analysis revealed that *BUD1* codes for a Ras-related small GTPase protein. Because *BUD1* deletion itself does not have any effect on growth rate, but only causes the cells to bud randomly, it is suggested that Bud1 acts only at the level of nonrandom bud site selection and not on further polarity development (17, 18). Interestingly, dominant positive (GTPase deficient) as well as dominant negative (GTP-binding deficient) mutants in *BUD1* also randomize their budding pattern with a strong tendency to form the first bud at the distal pole of the cell (42). In addition, a dominant positive mutation in *BUD1* suppresses the ability of nitrogen-starved diploid cells to undergo pseudohyphal growth (43).

BUD2 was isolated by screening a yeast genomic library for complementation of a *bud2* mutation that causes random budding (13). Chromosomal deletion or overexpression of *BUD2* again caused a random budding pattern without affecting growth rates of the strains. Bud2 can function as a GTPase-activating protein for Bud1 in vitro (13) and in vivo (44). *BUD2* was also isolated as *CLA2* (14) and *ERC25* (15) in screens for genes required for budding in the absence of G1 cyclins Cln1 and Cln2, proteins that regulate the transition from G1 to S phase in the cell cycle. Cln1 and Cln2 might play a role in bud site selection (14, 15), but this requires further experimentation.

BUD5, like *BUD1* and *BUD2*, is required for nonrandom budding (16, 17). Bud5 shares significant homology with Cdc25, a GTP-GDP exchange factor for Ras2 (16), and is required for suppression of a *cdc24* mutation by wild-type *BUD1* but not by a GTPase-deficient *bud1* mutant (18). This and other genetic evidence (16) suggests that Bud5 is a GDP-GTP exchange factor (activator) of Bud1 (44).

Bud1 Cycling and the Assembly of the Budding Complex

As mentioned above, the phenotype of mutants in the *BUD* genes suggests that Bud1 is critical for bringing together the proteins necessary for bud formation and the proteins that mark the incipient bud site (Figure 2). In fact, it has been shown recently (45) that Bud1 interacts with Cdc24 and Bem1, two components of the protein complex required for bud development (see below). Cycling of Bud1 between the GTP- and the GDP-bound forms affects these interactions: In the GTP-bound state, Bud1 binds preferentially to Cdc24, whereas in the GDP-bound state, binding to Bem1 is favored (45). We return to this point when discussing the protein complex that organizes bud formation.

PREPARING TO GROW

Once the site for bud emergence is defined, polarity establishment proteins are recruited to the chosen site. These proteins form a structure that organizes actin filaments into mobile cortical patches at the presumptive bud site and at the tip of the growing bud; this structure orients actin cables toward the growing bud (46–48). Orientation of the actin cytoskeleton is necessary for polarized delivery of building materials and for restriction of cell surface growth to the bud (11, 35a, 49, 50). Known members of the polarity establishment complex are the products of the *CDC42* (49), *CDC24* (52, 53), and *BEM1* genes (54).

CDC42

Cdc42 is a member of the *rho* (*ras* homologous) family of small GTP-binding proteins. *CDC42* is an essential gene originally isolated in a screen for mutants unable to bud at high temperature (49). The arrested cells grow in volume and continue to carry out DNA replication and nuclear division, hence becoming multinucleate (49). Such cells also display delocalized chitin deposition in the cell wall, a phenotype associated with loss of actin polarization (49). Immunofluorescence and immunoelectron microscopy demonstrated that Cdc42 localizes to the plasma membrane near secretory vesicles that accumulate at the site of bud emergence and at the tips and sides of enlarging buds. The Cdc42 staining was most pronounced near plasma membrane invaginations where cortical actin also was found (55); however, its overall staining pattern was different from that of actin (56). Although at permissive temperature *cdc42-1*^{ts} cells bud axially, overexpression of *CDC42*, as well as expression of another ts allele, *cdc42*^{W97R} in single copy, randomizes the budding pattern (57, 58), suggesting that Cdc42 may participate in recognition of the landmark that defines the incipient bud site.

Close homologues of Cdc42 are found in cells of other eukaryotes, such as *Homo sapiens*, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* (59–63). Some of these homologues are able to complement the *S. cerevisiae cdc42* mutation, which suggests that they may participate in a similar process.

Organization of Actin

A large amount of data has accumulated on proteins involved in polarity establishment and on actin and actin-binding proteins, but a huge gap remains in our knowledge of the regulation of actin polarization. In a newborn daughter cell that must grow isotropically to reach its mature size, actin is randomly distributed around the cell cortex as patches (46). Shortly before emergence of a bud, these patches congregate at a specifically selected site where the bud will emerge. During bud development, the actin patches are localized almost exclusively within the bud itself, and actin cables that form in the mother cell

orient along the mother-bud axis (46). As mentioned earlier, these cables are believed to serve as "highways" for delivery of new material carried by secretory vesicles to the growing site (64). When the bud is mature, the patches become randomized again. At cytokinesis, the patches reassemble at the mother-bud neck, where the division septum is constructed (46).

An elegant experiment showed direct involvement of Cdc42 in regulating actin assembly (65). Rhodamine-labeled actin monomers added to permeabilized yeast cells accumulated in buds to form cortical patches similar to those observed in vivo. Actin incorporation into the bud was stimulated by GTP-γS and was reduced by a mutation in *CDC42*. The impaired actin nucleation activity in the *cdc42* mutant was restored when a constitutively active (GTPase-deficient) Cdc42 protein was added to the assay (65). Lechler & Li (66) recently modified the assay to identify two sequentially acting protein factors required for actin nucleation: Bee1 (also called Las17), a protein homologous to mammalian Wiskott-Aldrich syndrome protein (67), and a new protein, Pca1. These results also show that the permeabilized cell assay is a fruitful approach to obtain more information about the function of other genes implicated in actin regulation.

One such gene with an elusive function is *BEM1* (bud emergence), which encodes a protein without any obvious enzymatic activity but containing two SH3 (*src* homology) domains found in proteins interacting with actin (54, 68). Bem1 localizes to the bud site at an early stage of bud assembly (unpublished data of Corrado & Pringle cited in reference 19) and binds to GDP-Bud1/Rsr1 (45). It also binds, in a Ca^{2+}-sensitive manner, to Cdc24 (69, 70), which is a GEF for Cdc42 (see below; 71). Bem1 also interacts with the protein kinase Ste20 (see below; 72) and, most interestingly, with actin (72). The physiological importance of the interaction between Bem1 and actin is not clear, but it seems that Bem1 does not directly regulate formation of actin structures. Rather, its function may be to localize the components of the polarity establishment complex represented by Cdc24 and Cdc42 to the preselected bud site (see above) and to stabilize their interaction with actin.

Mutants in the *BNI1* gene show defects in cytokinesis during vegetative growth (73, 74) and in polarized morphogenesis during mating (75). Bni1 is the yeast homologue of mammalian proteins called formins, which participate in morphogenesis (75). The NH_2-terminal portion of Bni1 binds to the activated form of Cdc42 and other rho-type G proteins, including Rho1, Rho3, and Rho4 (74, 75), all implicated in organization of the actin cytoskeleton. The *RHO1* product is a yeast homologue of human RhoA. The latter regulates actin-dependent cell functions such as cell motility, cytokinesis, cell adhesion, and smooth muscle contraction (76); at least the latter two functions are performed through regulation of myosin phosphorylation (77). Mammalian RhoA

can complement some functions of yeast *RHO1* (78). The COOH-terminal portion of Bni1 interacts with profilin (75), a highly conserved protein that stimulates actin polymerization (79). Thus Bni1 may be the common mediator through which rho-type G proteins induce actin polymerization, as predicted by Narumiya (76).

Reviews are available concerning actin structure and function (80), its regulation during the yeast cell cycle (64), and the role of rho-type G proteins in actin organization in higher eukaryotes (76, 81).

STE20

To date, the best-characterized target of Cdc42 is the protein kinase Ste20. Its homologue from mammalian cells, PAK (p21-activated protein kinase), is stimulated by binding of the mammalian homologue of Cdc42 (82). Loss of *STE20* function does not affect budding or the establishment of cell polarity in yeast (83); however, activation of Ste20 or its close homologue, Cla4, by GTP-Cdc42 is essential for localization of cell growth with respect to the septin ring and for cytokinesis (84). A *cla4 ste20* double mutant cannot undergo cytokinesis and is inviable (84, 85). In addition, Ste20 functions during mating in both the pheromone signaling pathway (83, 86) and the cell wall integrity signaling pathway (87) as well as in the transition to pseudohyphal and invasive growth (2). Ste20 mutants that can no longer bind Cdc42 are defective in pseudohyphal growth and are unable to restore growth of *ste20 cla4* mutant cells (85). Paradoxically, although the necessity for both Cdc42 (88, 89) and Ste20 (83, 86, 90) in the pheromone signaling pathway has been well documented, mutations in either of their mutual binding domains did not abolish transduction of the mating signal (85). Perhaps, in the mating response, the contact between Ste20 and its partners is facilitated by the scaffolding protein, Ste5 (72, 90). Alternatively, during pheromone signaling, Ste20 may be activated by a factor different from Cdc42. The latter is consistent with the observation that overexpression of dominant negative or constitutively active alleles of *CDC42* has no effect on the basal activity of the pheromone signaling pathway (89, 90), although overexpression of the constitutively active allele potentiates the response to the mating pheromone (89, 90).

Regulators

CDC24, *CDC43*, *BEM2*, and *BEM3* genes were also found to be necessary for the assembly of the bud (49, 71, 91). Cdc24 is a GEF for Cdc42, as mentioned above (71). It also binds GTP-bound Bud1 (45, 70), thus perhaps linking bud site selection and bud site formation.

CDC43 encodes a β-subunit of the geranylgeranyl transferase I that prenylates Cdc42, thus facilitating its attachment to the membrane (92, 93).

Mutations in *CDC42* that eliminate the isoprenylation site result in a nonfunctional product, which suggests that proper membrane localization is necessary for Cdc42 function (56, 94).

BEM2 and *BEM3* contain a rho-GTPase-activating protein (rho-GAP) homology domain, but only Bem3 is able to stimulate hydrolysis of GTP by Cdc42 in vitro (71). Another protein with rho-GAP activity for Cdc42 is Rga1 (Rho GTPase activating protein) (95). Loss of Rga1 activity causes cells to bud polarly and slightly increases activity of the pheromone signaling pathway, whereas its overexpression dampens it (95). Although genetic interactions between *RGA1* and *BEM3* with *cdc24*^{ts} support the possibility that they both function as negative regulators of Cdc42 in vivo (95), mutation of *BEM3* has not been reported to activate the pheromone pathway. At least one more protein with GAP activity on Cdc42 is expected to function in yeast because the *rga1 bem3* double mutation has a less severe phenotype than the constitutively active *CDC42*^{G12V} mutation (95). Several other genes encoding putative Rho-GAPs were identified in the yeast genome, but their function is unknown.

Phosphatidylinositol 4,5-bisphosphate (PIP_2) can regulate mammalian Cdc42 and Rho by functioning as their GEF in vitro (96). In *S. cerevisiae*, the putative phosphatidylinositol kinase Tor2, involved in organization of actin (97), appears to act upstream of Rho1 and Rho2 (98). However, in mammalian cells, phosphatidylinositol 4-phosphate-5-kinase was found to act downstream of Rho (99).

Other Players

BEM4 encodes a protein required for bud emergence at higher temperature (100). Because it interacts with constitutively active and dominant negative forms of Cdc42 as well as with Rho1, Rho2, and Rho4, its role in cell morphogenesis may be more general: e.g. it may play the role of a chaperone for small GTPases (100).

Boi1 and Boi2 (Bem one interacting) is another pair of proteins with unknown function that interact with both Bem1 and the GTP-bound form of Cdc42 (101). The stoichiometry of the components in this complex is crucial because overexpression of *BOI1* inhibits bud emergence, but this inhibition is counteracted by co-overexpression of *CDC42* (101). This complex may link the functions of Cdc42 and Rho3, because *RHO3* is an efficient dosage suppressor of *boi1 boi2* mutant, and, at the same time, overexpression of *RHO3* exacerbates the effect of overexpressed *BOI1* (101).

How the Machine Works

After seeing only a few parts of a complex apparatus, it is difficult to determine how it functions and how each component contributes. But a model, even if tentative, may help to orient us through the mass of data (Figure 3).

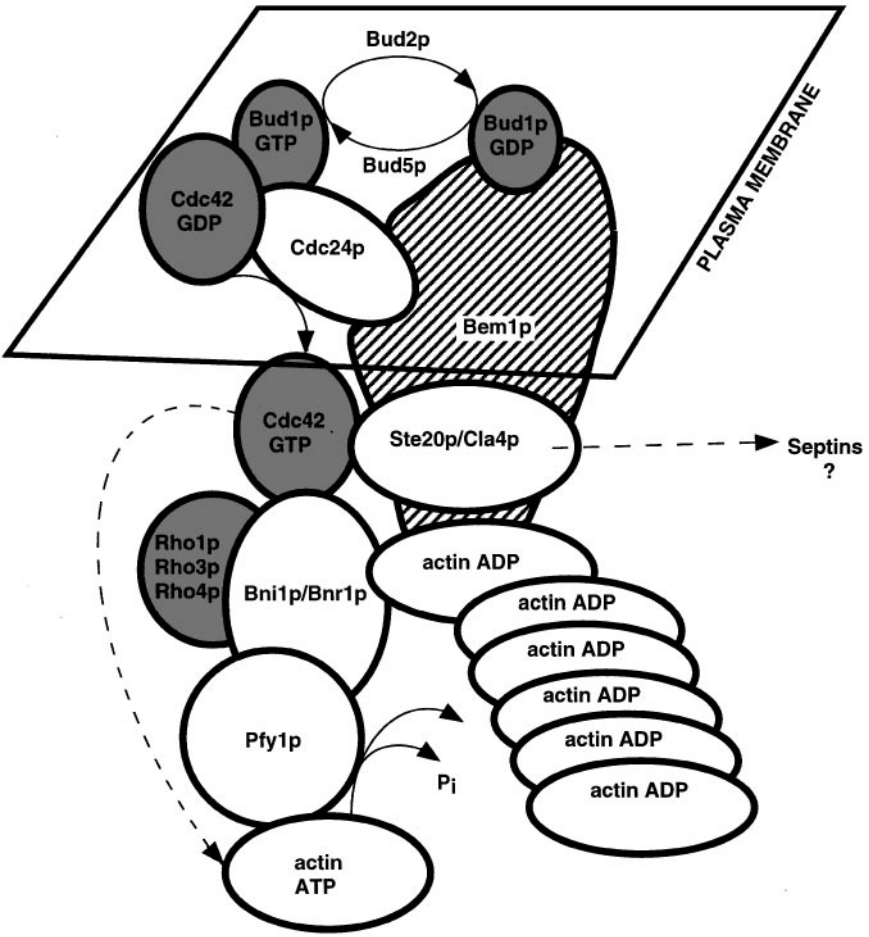

Figure 3 Schematic model for the organization of a bud site. *Solid arrows* indicate known or suspected conformational changes, and *dashed arrows* indicate hypothetical interactions. Small G proteins participating in the complex are shaded. Bee1/Las17, and Pca1, which probably function downstream of Cdc42 (66), are not depicted. For simplicity, proteins of the landmark complex have been omitted.

In the model depicted in Figure 3, the polarity establishment complex is targeted to the landmark defining the bud site by the activity of the small GTPase Bud1 (12). Because Bud1 itself is distributed rather uniformly over the cell surface, the landmark may bring about localized activation of Bud1 by affecting the positions and activities of its GEF Bud5 (16, 18) or its GAP Bud2 (13, 44). A loss of function of either Bud2 or Bud5, as well as any mutation of Bud1 that locks it in one state (either GTP- or GDP-bound) results in random budding.

Thus, for proper assembly of the polarity establishment complex, Bud1 must cycle between its GTP- and GDP-bound forms. Each of these forms has a different target: GTP-Bud1 binds to Cdc24, and GDP-Bud1 binds to Bem1 (Figure 3; 45, 70). In addition, Bem1 and Cdc24 bind to each other (Figure 3; 69, 70). What is the physiological consequence of all these interactions? Park et al (45) proposed the following model: In the first step, GTP-Bud1 would bind Cdc24, which in turn binds GDP-Cdc42 and Bem1 (Figure 3), thus recruiting the whole complex to the future bud site. In the second step, Bud2-induced hydrolysis of GTP-Bud1 to GDP-Bud1 would reorient Bud1 to Bem1. Consequently, Cdc24 released from the interactions with Bud1 and Bem1 would be able to activate Cdc42. Finally, Bud5 would regenerate GTP-Bud1 from GDP-Bud1, thus making it ready to start another cycle with a new load of Cdc24, Cdc42, and Bem1. Repeating the cycle would lead to an increased concentration of Bem1 and activated Cdc42 at the bud site. This model is at odds with results of Zheng et al (70), who observed that in vitro binding of Cdc24 to GTP-Bud1 inhibits both its intrinsic and Bud2p-stimulated GTPase activity and that binding of Bem1 or of Bud1 does not affect the GEF activity of Cdc24 toward Cdc42. If these behaviors are manifest in vivo, the cycle proposed by Park et al (45) would be blocked after the first step. Moreover, Bud1 seems not to be required for activation of Cdc42, only for its proper localization (see above). More work is necessary to understand the mechanism of bud site organization.

Activated Cdc42 binds Bni1 (Figure 3; 75), which through interactions with both actin and profilin can stimulate actin polymerization (Figure 3; 75, 79). Because it also binds actin, Bem1 may be positively involved in this process (Figure 3; 71). However, Bni1 is essential for actin polarization only during mating (75). During vegetative growth, its role may be at least partially supplanted by Bnr1 (102) or other proteins with related function. Bni1 interacts with the G protein Rho1 in vivo and in vitro and is a potential target of Rho1 (Figure 3; 74). At this point, it is not clear whether Rho1, similarly to mammalian Rho, functions downstream of Cdc42 (103, 104) or whether it acts independently.

Other targets of GTP-Cdc42 are the protein kinases, Ste20 and Cla4, which are required in a later stage of bud development for proper localization of cell growth with respect to the septin ring (Figure 3; 84).

Mating

As already mentioned, when a yeast cell is near another yeast cell of opposite mating type, it will grow a polarized projection along the pheromone gradient produced by the mating partner (105, 106). As in bud formation, the actin cytoskeleton, secretion, and new cell wall construction are polarized toward the tip of the projection (107) by the same polarity establishment molecules, which

include *CDC42*, *CDC24*, and *BEM1* (10, 54, 88, 108, 109). In this case, genetically determined instructions for axis formation are usually ignored, and the spatial cues are probably provided by the highest concentration of the pheromone-bound receptor, by Far1, (110, 111), and undoubtedly by other molecules. If these cues are missing because of the *far1-s* mutation, or because of the absence of the pheromone gradient when a cell is incubated in an isotropic solution of pheromone, the cell orients actin toward a regular (axial) bud site but still produces a mating projection and not a bud (112). It seems that the components of the polarity establishment complex that are common for both budding and mating can always recognize the bud site, but the presence of pheromone signal changes the situation inside the cell in such a way that only a projection can be formed.

BUILDING NEW CELL WALL

Once the budding machinery has been organized, actual growth of the daughter cell can begin, including addition of new cell surface (membrane and cell wall) and of intracellular material, both in soluble form and as organelles. We concentrate here on growth of the cell wall because it is directly involved in morphogenesis. The wall determines cell shape: Enzymatic digestion of the cell wall leads to the formation of spherical protoplasts; conversely, cell walls isolated after mechanical breakage of cells maintain the shape of the intact cell. Cell wall synthesis must be regulated in synchrony with the cell cycle. Before bud emergence, the mother cell wall is in a quiescent state. As the bud starts growing, wall synthesis is switched on; at daughter cell maturation, it is switched off. The composition of the cell wall is relatively simple, consisting of a few polysaccharides and of mannoproteins (113). The synthesis of each of these components must be under the control of the cell wall growth switches. Thus, control of wall growth can be studied at the molecular level by following biosynthesis of one of the main constituents. This approach has led to the finding that Rho1 is an essential regulator of the synthesis of the main structural component of the *S. cerevisiae* cell wall, $\beta(1 \rightarrow 3)$glucan.

A GTP Requirement for $\beta(1 \rightarrow 3)$Glucan Synthase

Although in vitro biosynthesis of $\beta(1 \rightarrow 3)$glucan in *S. cerevisiae* was reported as early as 1975 (114), conditions for an efficient transfer of glucose from UDP-glucose to an accepting glucan chain in the presence of membrane preparations were not determined until 1980 (115, 116). In these studies, GTP or some of its analogs were found to be potent stimulators of the enzymatic activity. The nucleotides were active at concentrations in the micromolar or submicromolar range, a level that may easily be present in vivo, suggesting the possibility that

they were physiological regulators of the synthase. Further progress was slow because localization of the synthase in membranes hindered its dissection and characterization. However, a later study of the effect of GTP on a number of fungal $\beta(1 \rightarrow 3)$glucan synthases (117) culminated in the finding that the enzyme could be dissociated by extraction with a mixture of salt and detergent into two components, one soluble (fraction A) and the other still membrane bound (118). Reconstruction of enzymatic activity required both fractions plus GTP. Thermal stability experiments in the absence or presence of the nucleotide suggested that GTP was interacting with the solubilized fraction. Similar results were later obtained with membrane preparations from *S. cerevisiae*. In this case, further extraction of the insoluble fraction with other detergents resulted in the solubilization of another fraction (fraction B), which, when added to fraction A, supported polysaccharide synthesis in the presence of GTP (119).

A GTP-Binding Protein Is a Component of $\beta(1 \rightarrow 3)$Glucan Synthase

Purification of fraction A from yeast by ion exchange and gel filtration chromatography led to cofractionation of GTP-binding activity, as measured by adsorption on nitrocellulose membranes, and ability to complement fraction B in a glucan synthase assay (119). The component of fraction A required for glucan synthesis was a GTP-binding protein (119). The elution profile of a sizing column suggested a molecular weight around 25,000, whereas a band at 20 kDa was photolabeled in the presence of [γ-^{32}P]GTP (119). However, microsequence analysis of the latter protein showed that this band was unrelated to the glucan synthase system (T Drgon and E Cabib, unpublished results). The labeling appears to have been spurious.

These results suggested that a small GTP-binding protein was involved in the activity of $\beta(1 \rightarrow 3)$glucan synthase and provided a rationale for the switching on and off of the enzyme during the cell cycle, presumably by alternating between the active GTP-bound and the inactive GDP-bound states (119). A genetic approach was needed to determine whether this GTP-binding protein was a new small G protein or one of those already detected in yeast.

Rho1 Is the Regulatory Subunit of $\beta(1 \rightarrow 3)$Glucan Synthase

In an attempt to identify the small G protein involved in glucan synthase activity, the reported phenotypes of mutants in known yeast G proteins were scrutinized. Only mutants in two pairs of closely related genes, those in the *RHO1-RHO2* pair (120) and in the *RHO3-RHO4* pair (121), showed a phenotype compatible with the expected defect. In both cases, conditional mutants lysed when brought to a nonpermissive condition, and the majority of cells susceptible to the lysis bore a small bud. It was reasoned that a cell incapable of switching glucan

synthase on at bud emergence would give rise to a bud with a defect in the wall, leading to cell lysis. Measurements of glucan synthase activity showed a normal enzyme in *rho4*Δ*rho3*^{ts} mutants. However, membrane preparations from *rho1*^{ts} mutants were clearly defective even at permissive temperatures and showed almost no stimulation by GTP (122). Addition of purified fraction A or recombinant Rho1 restored both activity and GTP stimulation (122, 123). Glucan synthase activity was also reconstituted by addition of recombinant Rho1 to fraction B, showing that Rho1 is the only active component of purified fraction A (122). Furthermore, when purified preparations of fraction A from wild-type and *rho1*^{ts} cells were subjected to SDS-polyacrylamide gel electrophoresis, a band at 24 kDa was found to be absent in the mutant. The same band was labeled by ADP-ribosylation with *Clostridium botulinum* C-3 exoenzyme, as was recombinant Rho1 (122). Rho1 is the only Rho protein from *S. cerevisiae* that is ADP-ribosylated by the C-3 exoenzyme (78, 124). From all this evidence it was concluded that Rho1 is the G protein present in fraction A that is necessary for glucan synthase activity.

On the Nature of the Direct Target of Rho1 in the Glucan Synthase System

To act as an activator of glucan synthase, Rho1 presumably must interact directly with the catalytic subunit of glucan synthase or with a protein associated with it. Fraction B is a crude preparation that contains many proteins. However, glucan synthase has been purified extensively from solubilized membranes by the product entrapment procedure (125). These preparations were enriched in the product of the *FKS1* gene, which, together with its homologue, *FKS2* (126), has been implicated in the activity of glucan synthase (127, 128). Mutants in *FKS1* were first isolated in a screen for hypersensitivity to immunosuppressants (129), and again, under the name *etg1*, in a screen for strains resistant to glucan synthase inhibitors (127). Null (*fks1*Δ) mutants show a decrease in glucan synthase activity (128) and in incorporation of glucose into β $(1 \rightarrow 3)$glucan in vivo (130); double null (*fks1*Δ*fks2*Δ) mutants are inviable (126). Furthermore, immunoprecipitation of Fks1 or Fks2 from purified preparations of the synthase resulted in coprecipitation of enzymatic activity (126, 131). These results led to the conclusion that Fks1 and Fks2, large hydrophobic proteins with 16 putative transmembrane domains (126, 128), are essential components of the glucan synthase system. It could not be established conclusively, however, whether they represent the catalytic subunit of the synthase, because even the most purified preparations contained other proteins. Fks1 and Fks2 seem to have different, if overlapping, functions because they are regulated differently. During vegetative growth, Fks1 is preferentially expressed (126), whereas Fks2 is required for sporulation (126). Also, Fks2 is induced by Ca^{2+} as well as by pheromones, in both cases in a calcineurin-dependent fashion (126).

Although it has not been shown that Fks1 or Fks2 interacts directly with Rho1, they appear to be part of the same complex. Rho1 copurifies with Fks1 upon product entrapment of glucan synthase (123, 131), and immunoprecipitation of Fks1 results in coprecipitation of Rho1 (123, 131). Consequently, although it has not been proven that Fks1 and Fks2 are direct targets of Rho1, they are strong candidates.

Localization of the Glucan Synthase Components and a Scheme for Their Interaction

It has long been known that $\beta(1 \rightarrow 3)$glucan synthase activity is localized to the plasma membrane (115). Because Rho1 is essential for activity, both the G protein and the catalytic components must be at that location. The presence of a prenyl group on Rho1 (132) favors its binding to the membrane. At bud emergence, the glucan synthase complex would be expected to be at the bud organization site, to start cell wall formation in the new bud. In fact, Yamochi & coworkers (132) detected Rho1 at the bud site, in the same general area where actin is localized, by immunofluorescence, whereas Qadota et al (123) used a similar methodology to show Fks1 at the same site. Both proteins appear to relocalize to the neck between mother and daughter cell at septum formation (123, 132). These findings suggest a simple scheme for the interaction of the glucan synthase components (Figure 4). The catalytic portion may be present in the plasma membrane in an inactive form (Figure 4, where the closed "gate" symbolizes the inactive state). When recruited to the bud site (Figure 4), it finds Rho1, which by exchanging GDP for GTP has undergone a conformational change that enables it to bind to the catalytic complex. This binding, in turn, changes the conformation of the catalytic subunit (gate open), uncovering the active site, to which UDP-glucose can now attach. Glucan synthesis ensues, with simultaneous extrusion of the polysaccharide into the extracellular space, as was formerly demonstrated for chitin (133). The possibility of an indirect interaction with actin, as discussed in a previous section, is also indicated (Figure 4).

The Regulation of Rho1

Candidate regulators of Rho1 have been found in yeast, although there is evidence for only some of these proteins that they have the expected physiological function (Figure 5). Two GEFs with apparently overlapping functions were detected: Rom1 and Rom2 (134). A double null (*rom1*Δ *rom2*Δ) mutant showed a phenotype similar to that of *rho1*Δ strains, i.e. the mother cell arrests with a small bud (134). The question of which of several candidates may be physiological GAPs for Rho1 is not yet resolved. The GAP domain of Bem2 is active on Rho1 in vitro (69), but the phenotype of cells containing a *bem2*Δ mutation

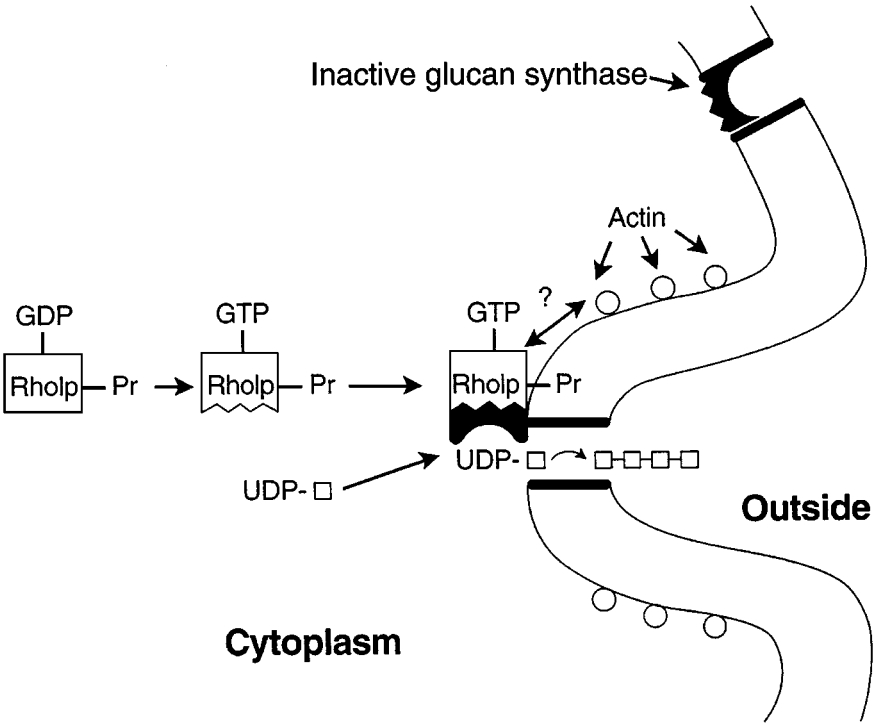

Figure 4 Scheme for the regulation of $\beta(1 \rightarrow 3)$glucan synthesis at the site of bud emergence. Actin filaments are shown in section around an invagination of the plasma membrane encompassing a small portion of the bud site (55). The GTP-GDP exchange in Rho1 is shown as taking place in the cytoplasm, but it could occur on the membrane. The small squares attached to UDP are glucosyl units. *Pr*, prenyl group. Adapted from reference 122.

(large and multinucleate) (135) is difficult to interpret as the consequence of an inability to inactivate Rho1. It is also unclear why overexpression of *RHO1* and *RHO2* would suppress rather than exacerbate a *bem2* defect (135). Another candidate GAP is Sac7, although in this case the demonstration of in vitro activity is not convincing (98). In the course of purification of Rho1 (fraction A), a fraction with apparent GAP activity on Rho1 was isolated (119). The activity is still present in extracts from strains with mutations in putative GAPs (J Drgonová, CSM Chan, E Cabib, unpublished data), such as Bem2 (135), Bem3 (71), Dbm1/Rga1 (136), and Lrg1 (137). Finally, a gene for a GDI was cloned and the corresponding protein purified and shown to act on Rho1 (138). There is, however, no phenotype associated with disruption of this gene (138).

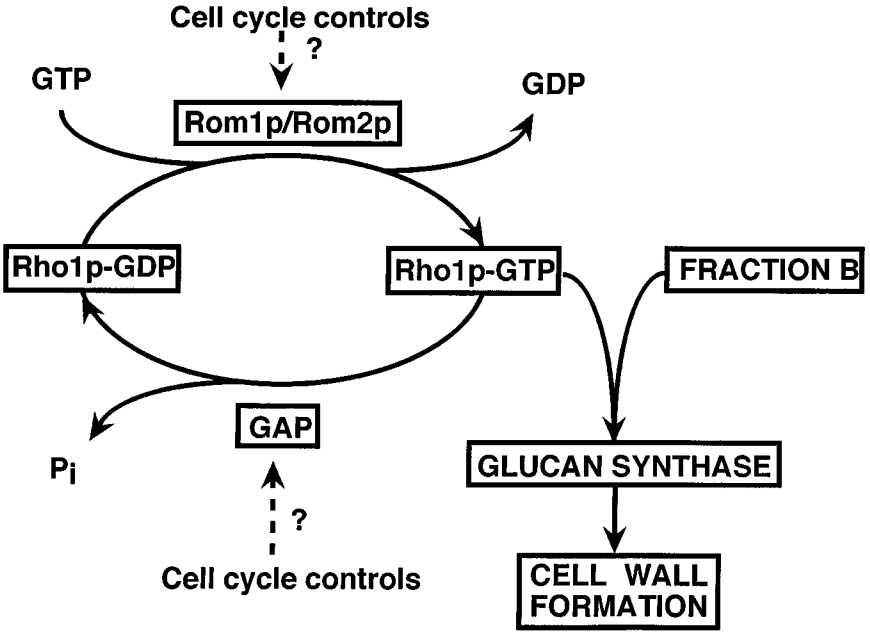

Figure 5 Scheme for the regulation of Rho1 activity and $\beta(1 \rightarrow 3)$glucan synthesis. For explanations see text.

The effects of the different regulators are summarized in Figure 5. Clearly, the opposing actions of a GEF and a GAP can switch Rho1 activity and therefore glucan synthase and cell wall biosynthesis, on and off. That in the glucan synthase system only GTP-Rho1 is active was demonstrated with the help of the above-mentioned purified GAP activity (119). Furthermore, in cells containing a dominant active allele of *RHO1*, glucan synthase was active in the absence of added GTP (123). However, GAPs and GEFs represent only one aspect of regulation. Glucan synthase activity must be strictly regulated in time, in synchrony with the cell cycle. Therefore, the cell cycle machinery may modulate the GAPs or GEFs or both (Figure 5).

Other Functions of Rho1

In addition to its direct effect on $\beta(1 \rightarrow 3)$glucan synthase, Rho1 has other functions. Both genetic (139) and biochemical (140) evidence demonstrates that Rho1 can interact with and activate the protein kinase Pkc1. This enzyme in turn activates a MAP kinase cascade, which functions to maintain integrity of the cell wall (141–144). The nature of the ultimate target(s) of the cascade

is not clear; however, one of its effects is to increase transcription of *FKS2* at high temperatures (140). A *pkc1* mutant shows a partial defect in components of the cell wall and exhibits synthetic lethality with some members of the *KRE* gene family (145). The latter are involved in the synthesis of $\beta(1 \rightarrow 6)$glucan, which plays a central role in the cross-linking of the different components of the yeast cell wall (146). A recent report (147) identified SBF, a transcription factor that regulates the transition between G1 and S in the cell cycle, as another target of the Pkc1-regulated MAP kinase cascade. In this signaling pathway, the Cdc28 kinase seems to work as an upstream regulator of Pkc1 (148, 148a). So far, however, it is not known whether Rho1 mediates the effect of the Cdc28 kinase on Pkc1.

A *pkc1*Δ null mutant lyses with a small bud, a phenotype similar to that of *rho1*^{ts} mutants (132, 144, 148b). However, the *pkc1* defect is suppressed by osmotic protectors (141), whereas that of *rho1* mutants is not (132; E Cabib, J Drgonová, unpublished data). If the only other target of Rho1, in addition to Pkc1, were glucan synthesis, osmotic protectors should prevent lysis, just as they do for protoplasts that completely lack a cell wall. A possible function of Rho1 in actin organization (see above) might explain these results, because actin mutants show some lysis that is not suppressed by sorbitol (149). Another issue with the phenotype of *pkc1* mutants arises in the above-mentioned case of SBF. Because the latter is required for the G1 to S transition, and if the function of SBF depended solely on the activity of Pkc1, *pkc1* mutants should be blocked in G1. However, they proceed to bud before they lyse. Together with the finding that overexpression of Pkc1 suppresses a null mutation of *SW14*, which encodes a component of SBF (148a), these results suggest the existence of two pathways for budding, one that requires SBF and the other Pkc1 activity. Despite these complications, it seems safe to conclude that Rho1 regulates cell wall synthesis both directly through glucan synthase and indirectly through the Pkc1-dependent MAP kinase cascade.

Rho3 and Rho4, Two Proteins Implicated in Bud Growth

RHO3 and *RHO4* encode small G proteins that appear to have overlapping functions (121). Disruption of *RHO3* results in very slow growth, whereas null mutants of *RHO4* have no discernible phenotype. However, *RHO4* is a dosage suppressor of the *rho3* null defect, and a double deletion of both genes is lethal. The phenotypes of mutants are somewhat variable. When a strain harboring deletions of both *RHO3* and *RHO4* as well as a plasmid carrying *RHO4* under control of a *GAL7* promoter was shifted from galactose to glucose medium, after many hours most of the cells were found to be lysed with a small bud (150). However, when cells with a *rho4* deletion and a temperature-sensitive *rho3* mutation were shifted to the nonpermissive temperature, they ended up as large

cells in which actin was delocalized (151). Matsui and his coworkers concluded that *RHO3* and *RHO4* are involved in maintaining cell polarity during bud growth (150, 151), but it is not clear how they accomplish this function. There are tantalizing interactions with other genes. Thus, overexpression of *BEM1* or *CDC42*, both participants in bud site organization (see above), suppresses the *rho3*/*rho4* defect (150). As mentioned above, overexpression of *RHO3* or *RHO4* suppresses the *boi1* or *boi2* mutation, which has a similar phenotype to *rho3* (101, 152). Finally, *rho3* is synthetically lethal with mutants of *SEC4*, whose product is a Rab-type G protein involved in the fusion of vesicles with the plasma membranes (151). To understand these interactions, it is necessary to determine the targets of Rho3 and Rho4.

SMALL G PROTEINS MUSINGS

As has been described here, both the selection of a bud site and its organization are intricate processes that require the formation of complexes that include multiple proteins. Most of these proteins have been identified by the use of screens for interacting genes, such as suppression of a mutation by high-copy expression of another gene, two-hybrid interaction, and synthetic lethality. Whereas these techniques have been invaluable for the identification of proteins potentially participating in a physiological function, they are not without flaws. For instance, it may happen that the two-hybrid procedure recognizes two proteins that are part of the same complex but do not interact functionally or physically or that the simultaneous presence of two unrelated mutations makes the cell so sick that "synthetic" lethality ensues. Furthermore, genetic methods do not indicate the nature of the interaction among different proteins. Their use has often led to very complex schemes in which the putative participants in a cellular process are connected by arrows whose meaning is difficult to assess. One striking example of this situation is found in the recent cloning of two genes found in so many screens that they were named *ZDS* for zillion different screens (153). Some of these difficulties are caused at least in part by the fact that such complex processes as cell polarization and morphogenesis are not strictly sequential or only so to a certain extent (and in those cases genetic determination of epistasis is of great help). In contrast, metabolic pathways are usually linear processes in which each product is the precursor of the next one, which explains the spectacular success of genetics in the elucidation of metabolic routes. Therefore it is very important to complement genetic data with results obtained by other methodologies. These strategies include localization of gene products by immunofluorescence; in vitro binding of heterologously expressed proteins; and, in the case of G proteins, biochemical measurement of the activity of a putative GAP, GEF, or GDI. A more ambitious

methodology is needed, however—specifically, in vitro reconstruction of the system under study. This approach has been very successful in the analysis of vesicular transport and secretion (154), which involve events of a complexity comparable to those discussed in this review. A beginning has been made by Lee and coworkers (65) with the use of permeabilized cells to study actin organization (see above). Further development of such systems, though difficult, could be very productive.

Despite the problems and uncertainties, great progress has been made in understanding the mechanism of bud site selection and organization, especially in the identification of major players in these complex events. Two of them are small G proteins, Bud1 for bud site selection and Cdc42 for bud site assembly. In their absence, the corresponding process does not take place. What determines their localization and how they interact with their presumptive targets is not clear. More work is also needed to establish how their cycling between active (GTP-bound) and inactive (GDP-bound) forms is integrated in the processes they control.

The function of Rho1 in cell wall morphogenesis as represented by the synthesis of $\beta(1 \rightarrow 3)$glucan is easier to understand because here the immediate target, the enzyme $\beta(1 \rightarrow 3)$glucan synthase, is known. In fact, so far this case is unique because a small G protein directly signals the system that gives rise to the ultimate product of the regulatory cascade, here the cell wall. This mechanism for regulation of $\beta(1 \rightarrow 3)$glucan synthase seems to be quite general in fungi, because fractions with similar activity to fraction A from *S. cerevisiae* were isolated from several organisms (117). In *Schizosaccharomyces pombe*, a homologue of *S. cerevisiae* Rho1 is the synthase regulator (155). On the other hand, we know very little about the upstream regulation of Rho1 that ultimately must synchronize cell wall synthesis with other events of the cell cycle.

Homologues of the three G proteins discussed here are known to exist in mammalian cells, where they participate in many cellular processes, such as formation of stress fibers (156), lamellipodia (157), and filopodia (158, 159). The mechanisms of action of these proteins may be expected to share similarities in both mammalian organisms and yeast. The latter is more amenable to genetic approaches, whereas mammalian cells are accessible to certain techniques, such as microinjection, that cannot be used with yeast. The results obtained with one type of cell may therefore complement those found with the other and help in the elucidation of mechanisms for polarization and morphogenesis, which are processes common to all organisms.

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