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Molecular Basis of Cell Integrity and Morphogenesis in *Saccharomyces cerevisiae*†

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INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* has long proved to be a useful model for the study of many diverse basic aspects of cell biology. Particularly in recent years, it has provided a wealth of information about new gene products involved in the establishment and maintenance of cell morphogenesis. Although morphologically simple in appearance, baker's yeast is endowed with many of the same basic biochemical components that determine cell shape and cell polarity as those found in higher eukaryotes. Precisely because of its simplicity and amenability to manipulation by molecular and classical genetics, it has provided a valuable system for understanding how complex biological processes occur in the living cell.

As in all fungal cells, morphology in *S. cerevisiae* is at least partly determined by the cell wall, a rigid structure that affords mechanical protection, dictates cell shape, and modulates the selective uptake of macromolecules. Without this barrier, the cell would be unprotected from environmental changes and would lyse because of osmotic differences with the surrounding medium. In the present work, we focus on our current understanding of the composition of the cell wall and the biosynthetic pathways that lead to its formation, how cell wall dynamics are regulated at genetic level, and how this structure adapts to the physiological requirements of the cell throughout the different stages of the life cycle in the budding yeast.

A scheme of the *S. cerevisiae* life cycle is depicted in Fig. 1A. Micrographs from the different stages are shown in Fig. 1B. Vegetative reproduction of the typical oval cells is achieved by budding. The bud first appears at the end of G_1 phase, after the START landmark in the cell cycle. The bud grows through the S phase, when DNA replication takes place in the mother cell, and reaches its final dimensions at the time of mitosis. After mitosis, the cell cycle culminates with the formation of the septum that eventually allows the separation of the daughter from the mother cell (i.e., cytokinesis). Yeast cells divide such that the resulting cells have different sizes, and hence the released daughter must increase its size before it starts a new cycle (183), causing a delay in the commitment of budding compared with that of the mother cell.

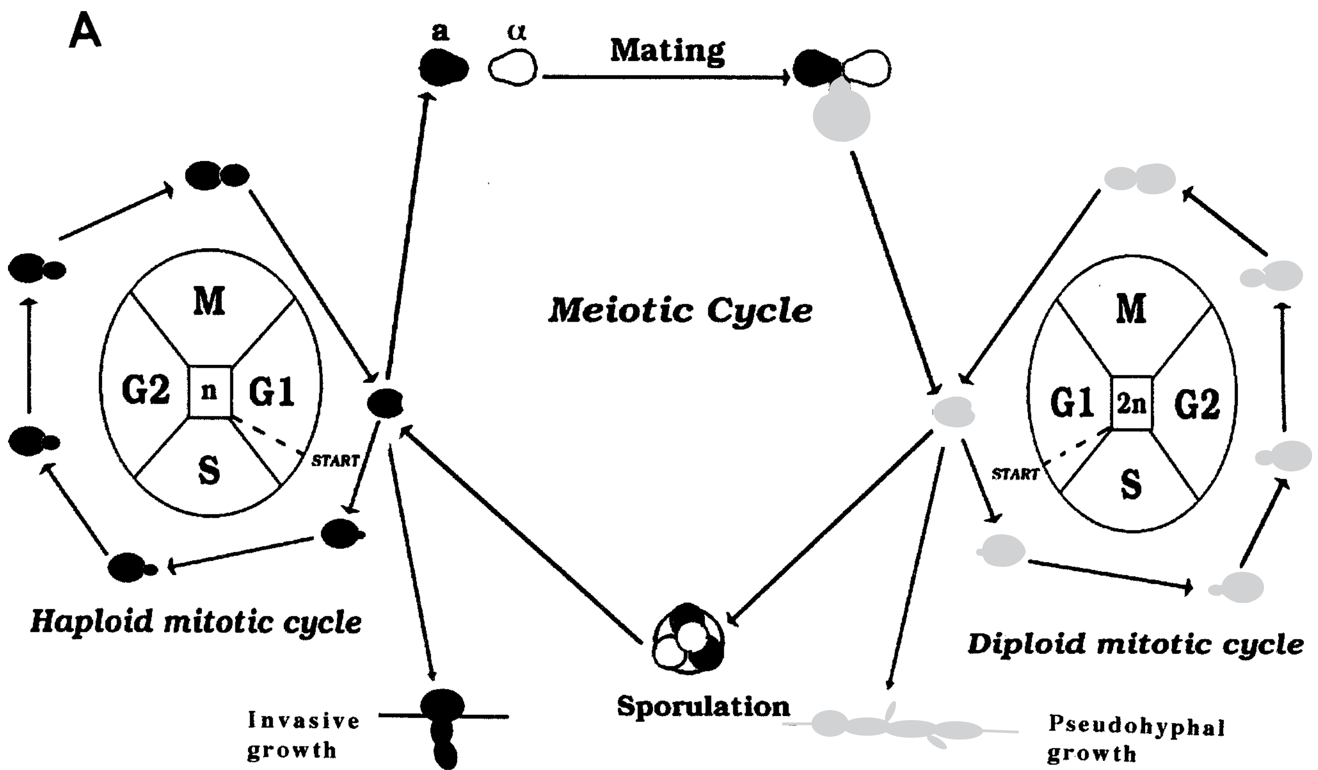
Haploid and diploid mitotic cycles are very similar; however, one notable difference is the preferred sites used by the different cell types for new-bud emergence. Haploid yeast cells are of two sexual types: **a** and α. Each cell type is able to produce a small peptide (the mating pheromones **a**-factor and α-factor) that leads cells of the opposite mating type to both arrest in G_1 (237) and form projections on one edge of the cell (12). The resulting pear-shaped cell is termed a shmoo. Mating cells interact by the tips of their projections, leading to cell fusion and, later, karyogamy. Finally, a bud emerges from the bridge that connects the mating cells. The **a**/α diploid will reproduce vegetatively by budding if the appropriate nutrients are available. However, under appropriate conditions of nitrogen and glucose starvation, it will undergo meiosis and sporulation, thereby producing four haploid cells: two **a** and two α. Ascospores are round, and their wall is chemically different from that of vegetative cells. The cluster of four spores, grouped in a tetrahedral arrangement, is surrounded and protected by an external envelope, which constitutes the ascus wall (for reviews on the budding yeast life cycle, see references 55, 191, and 350).

More recent findings have pointed to additional cytological patterns in *S. cerevisiae*, such as pseudohyphae (166, 242, 271) and agar-invasive cells (363) (Fig. 1A). Pseudohyphal growth occurs only in diploid cells under conditions of nitrogen limitation. The cells become elongated and grow in unipolar fashion to originate chains (166). In contrast to budding in the normal yeast form, cell division in pseudohyphal growth is symmetrical, and thus mother and daughter cells undergo the next cell division round synchronously (242). The other reported cytological pattern—invasive growth—occurs in certain haploid strains even in rich media. It also relies on the development of chained cells, although each cell remains ellipsoidal (363).

In spite of its apparent rigidity, the cell wall must be a very dynamic structure since it is susceptible to the many modifications that occur at different stages of the life cycle (29, 115). Substantial changes in the composition and/or structure of the cell wall take place during bud emergence, bud (or mating-projection) growth, septum formation, cell separation, cell fusion of mating partners, spore formation, and spore germination. These alterations must be controlled by a strict regulation of the balance between the synthetic and degradative pathways for the polymers that constitute the cell wall and by the directed and targeted secretion of both active enzymes and cell wall components to sites where growth is required. Furthermore, the processes involved in cell wall synthesis respond to cell cycle controls and to environmental signals such as nutrient levels or mating pheromones. A vast array of new genes coding for enzymes involved in cell wall dynamics and their regulation has been described in recent years. This information has provided new insights into how cell wall synthesis is directed and polarized in the growing bud, a process that relies on the actin cytoskeleton. Our aim with this survey is to offer an overall view of the cell wall synthetic and degradative machinery and the pathways that may govern cell wall formation and morphogenesis. We are very aware that such a view is still very limited and focuses mainly on vegetative growth, since much less is known about morphogenesis at other times of the life cycle, for example during mating and meiosis. Therefore, most of what follows will be devoted to morphogenesis in the vegetative cycle, placing special emphasis on bud emergence and growth.

CELL WALL MOLECULAR ARCHITECTURE

The cell wall of *S. cerevisiae* basically consists of three elements, β-glucan, chitin, and mannan, that are interconnected. Their distribution is depicted in Fig. 2. 1,3-β-Glucan is the main component of the cell envelope, forming the constructional network in which mannoproteins (mannan) are anchored. Chitin is present in small amounts in vegetative cells and is restricted almost entirely to the ring that encircles the septum in budding cells or the bud scar in mother cells. As shown in Fig. 2, the cell wall appears as a layered structure under the electron microscope, exhibiting a fibrillar outer layer, very rich in mannoproteins, and an amorphous inner layer (200). The inner layer itself may also consist of two different zones: an inner, less amorphous one, close to the membrane and rich in proteins, and an outer one, facing the mannan-rich area, which seems to contain an important pro-



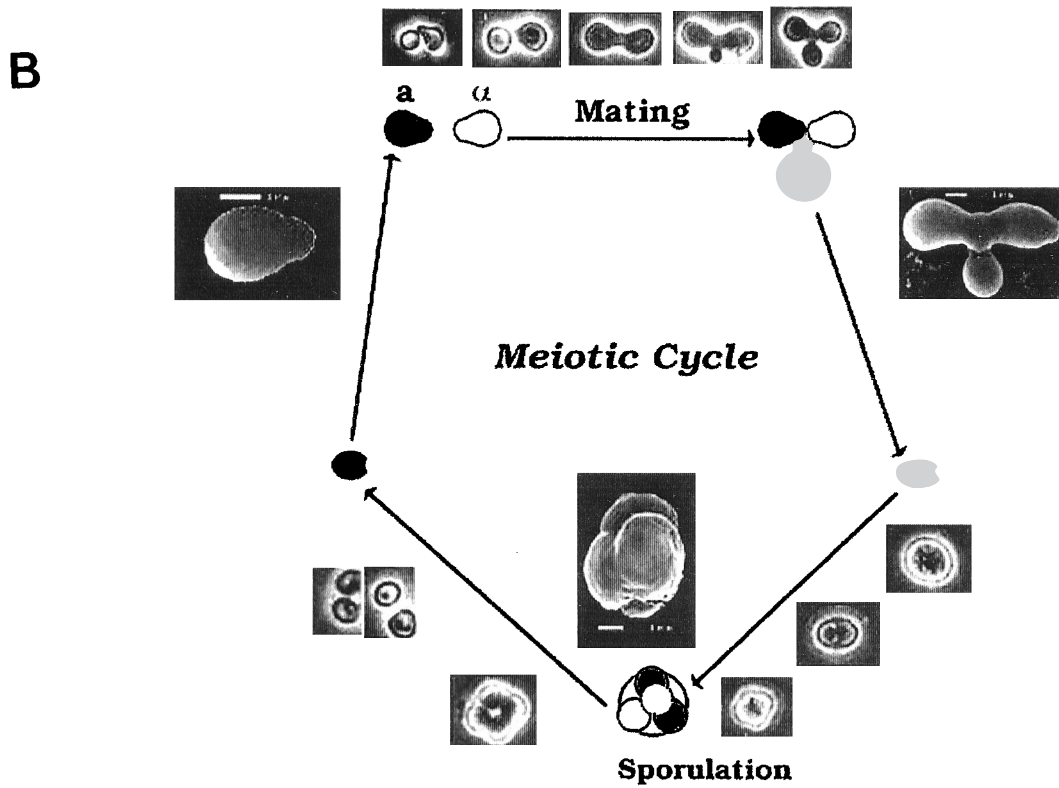


FIG. 1. Scheme (A) and micrographs (B) of the life cycle of the budding yeast *S. cerevisiae*. The bars in panel B represent 1 μm.

portion of 1,6-β-glucan (200, 239). A small amount of chitin is encompassed within the bulk of glucan.

For the study of cell wall composition, chemical fractionation of cell walls based on their different solubility upon treatment with alkaline solutions has been performed (13, 200, 239). This yields mannoproteins (at least 25% of the wall weight) and three classically known types of glucan: (i) an amorphous fraction that is soluble in alkali (25% of the wall weight) (143), consisting mainly of glucose chains of up to 1,500 residues bound by 1,3-β-*O*-glycosidic bonds; (ii) an alkali-insoluble acetic acid-soluble fraction (approximately 5% of the wall weight) which, in contrast, is formed mainly of glucose monomers bound by 1,6-β-*O*-glycosidic bonds (142, 286); and (iii) a fibrillar alkali-insoluble acetic acid-insoluble fraction (35% of cell wall weight) which seems to be responsible for cell wall rigidity (287). This third fraction consists mainly of 1,3-β-glucan, and its alkali insolubility is explained by the recently reported existence of a 1,4-β linkage between the terminal reducing residue of a chitin chain and the nonreducing end of a 1,3-β-glucan chain (234). In addition, it has been shown that chitinase treatment of the alkali-insoluble glucan renders it soluble (299) and that alkali-soluble β-glucan serves as a precursor in vivo for the alkali-insoluble fraction (181). Therefore, now that the interconnections between polysaccharides are being unveiled, the classical distinction between alkali-insoluble and alkali-soluble β-glucan fractions may be largely artificial.

Extraction of certain cell wall mannoproteins by nondestructive chemical procedures requires enzymatic digestion. Recent reports have proposed the existence of mechanisms that would be responsible for anchoring the mannoproteins to the cell wall (112, 431). These mechanisms promote cross-linking between the mannoproteins and 1,6-β-glucose chains, which are in turn attached to the 1,3-β-glucan chains. Therefore, mannoproteins appear to play an active role in cell wall morphogenesis.

As one might expect, this composition is subject to quantitative variations during the development of a new bud, which virtually lacks chitin and in which mannoprotein expression may be different from that of a mature cell. Such variations may be reflected, for instance, in the permeability of the cell wall, which increases in the initial stages of the budding process (113). The chemical composition of the cell wall is apparently uniform around the ellipsoidal cell with the exception of the septum and the chitin ring that encircles it, which show a different chitin/glucan ratio (Fig. 2; also see below).

Upon exposure to mating pheromone, the cell wall at the mating projection probably shows a slightly different composition, as deduced by analysis of the mannoproteins from α-factor-treated *MAT***a** cells (189, 265, 267). Changes at this stage have not only been detected in mannan; glucan seems to increase its proportion of 1,6-β links, and the amount of chitin increases about threefold (142, 388). Thus, the mating phenomenon involves localized growth of the cell wall, implying the activation of glucan and chitin synthesis and the conformation of a different pattern of wall proteins. This probably leads to an enrichment of those required for cell fusion, such as agglutinins (266) or *FUS1* and *FUS2* gene products (424).

If the controls that direct the changes in cell wall composition in mating cells constitute quite an unexplored field, even more unfamiliar to us is the regulation of ascospore wall formation. Spores show a significant amount of lipids and an increased quantity of proteins in their wall (43). These proteins, rich in dityrosine, are probably specifically expressed during sporulation (42). Chitosan, the deacetylated derivative of chitin, is present in large amounts (41). Glucan appears more branched than in vegetative cells (142, 443). These components are distributed in an apparent tetralayered configuration (41), and this results in a highly resistant structure, mostly because of the dityrosine-rich protein and chitosan layers (42).

Below, we discuss a series of biosynthetic enzymes and the possible regulatory pathways involved in the generation and maintenance of the cell wall, although the assembly of such a complex structure probably requires coordinated interactions and the regulation of a greater number of components than those currently known. A recent search for mutants presumably defective in cell wall assembly has opened the possibility of finding such new components (353). For a recent review about yeast cell wall assembly, the reader is referred to reference 231.

CELL WALL BIOSYNTHETIC PATHWAYS

Chitin

Chitin is a linear polymer of β-1,4-*N*-acetylglucosamine that accounts for only a small percentage of the cell wall dry weight in vegetative cells. The linear chains associate into microfibrils by forming hydrogen bonds between residues of adjacent chains. This leads to a crystalline structure that constitutes a structural component of the cell wall that is essential for the yeast cell (393). For other reviews about chitin biosynthesis in yeasts and fungi the reader is referred to references 49 and 66.

Chitin deposition. In *S. cerevisiae*, chitin is found mostly in the bud scar, a craterlike structure on the surface of the mother cell after cell separation (13, 60) (Fig. 2) that is easily detectable by Calcofluor white staining (349). The formation of the bud scar during the budding cycle occurs in several steps (68). As soon as the bud emerges, a chitin ring is formed at the base of the growing bud, and the deposition of chitin at this location continues as the bud grows to the size of the mother cell. Then, chitin synthesis occurs in a centripetal fashion by accompanying the plasma membrane as it invaginates to initiate the separation between mother and daughter cells. Thus, a very thin electron-translucent disk structure, the so-called primary septum, is formed (Fig. 2B, panel 1). The deposition of additional cell wall material (β-glucan and mannan) from both sides of the primary septum produces the secondary septum (Fig. 2B, panel 2). Finally, the two cells separate in an asymmetrical fashion. A birth scar is unconspicuous on the surface of the daughter cell and a more conspicuous structure, the bud scar, remains on the surface of the mother cell (Fig. 2C).

Besides the bud scar, the lateral wall also contains a small amount of uniformly dispersed chitin (Fig. 2), which becomes apparent after staining with wheat germ agglutinin-fluorescein isothiocyanate complex (301). Newborn daughter cells do not show detectable amounts of chitin, which is present only in the mother cells (371, 393). It is not clear whether this incorporation of chitin takes place only the first time that a cell becomes a mother or occurs repeatedly with each new budding.

During the process of polar elongation, which takes place upon the conjugation of two haploid cells of complementary mating types, or in response to incubation with the corresponding sexual pheromone (**a** or α factor), chitin is also synthesized (388). Chitin deposition occurs at the subapical portion of the shmoo tip, as can be readily observed after Calcofluor white staining (267).

Under sporulation conditions, diploid cells are converted into asci containing four meiotic products or ascospores (Fig. 1B). Late in this developmental process, the four nuclei are encircled by a double membrane (55) and cell wall components are deposited between the two membranes. Early studies suggested that the spore wall would contain glucosamine. Further-

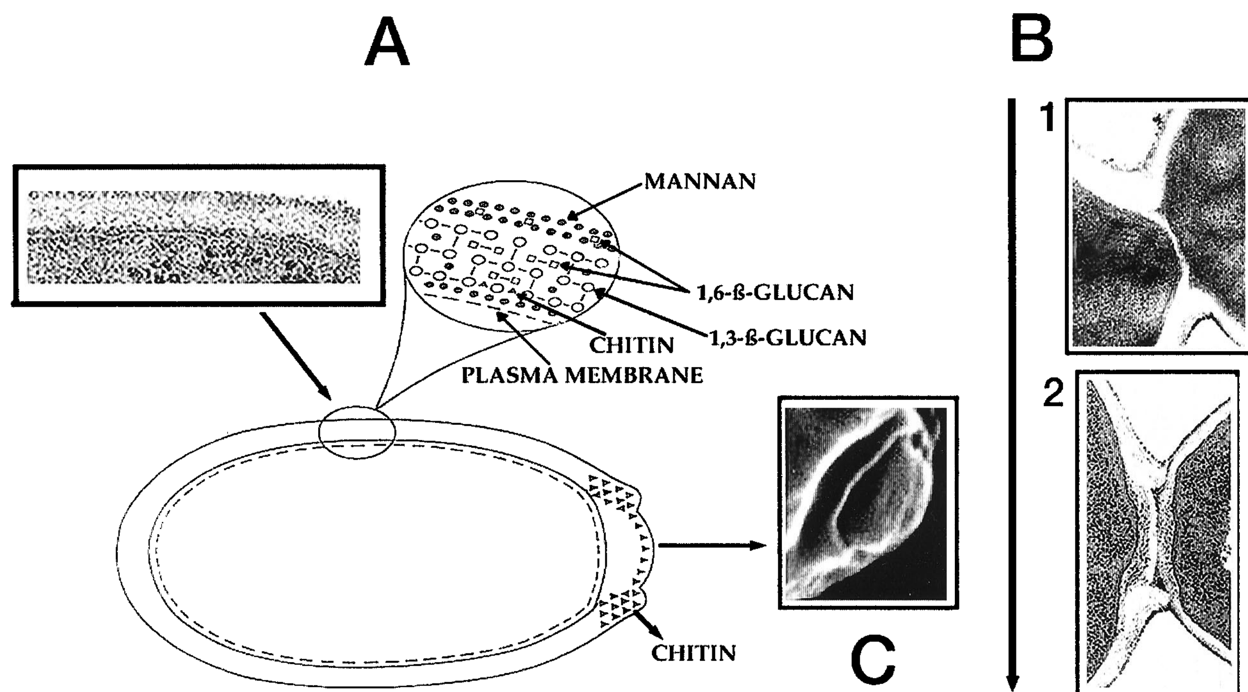


FIG. 2. (A) Model of cell wall structure in budding yeast. (B) Thin-section electron micrographs of the primary septum (panel 1) and of the septum area before cell separation (panel 2). (C) Scanning electron micrograph of the bud scar.

more, walls from glucosamine auxotrophic mutants lacked this aminosaccharide and such mutants produced abnormal spores (19, 447). More recently, it has been shown that the spore wall consists of four layers, the second one made of chitosan, a deacetylated derivative of chitin (41). According to the results of a study of chitosan biosynthesis in *Mucor rouxii* (104), it can be assumed that chitosan would be originated in two concomitant processes, i.e., the synthesis of chitin and the deacetylation of *N*-acetylglucosamine residues. Thus, chitin synthesis also occurs during sporulation.

The following sections address the enzymes and genes involved in chitin synthesis. The functions and possible regulatory mechanisms of the different synthases are also described. Table 1 gives a summary of these aspects.

Chitin synthase I: genes involved and function. Chitin synthase I activity (CSI) is a membrane-bound enzyme that catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) residues from UDP-GlcNAc to a growing chain of chitin. This is the major CS detected in *S. cerevisiae* and represents about 90% of the in vitro activity measurable in a wild-type strain (for a review, see references 57 and 64). CSI shows an absolute requirement for a divalent cation: Mg^{2+} or Mn^{2+}. It is uniformly

TABLE 1. Genes involved in cell wall chitin synthesis

Gene (synonym)	Essential	Chromosomal location	Proposed enzymatic function	Time or place of chitin synthesis	Other comments	Reference(s)
CHS1	No	15	Structural gene for CSI	In cytokinesis (repair function)	Transcription activated by mating factor	9, 51, 65, 67, 82, 336
CHS2	No	2R	Structural gene for CSII	Primary septum	Transcriptional and posttranslationally regulated	50, 82, 309a, 336, 385, 393, 401
CHS3[a] (*CSD2*, *DIT101*, *KTI2*)	No	2R	Catalytic component of CSIII	Budding neck ring and lateral wall in vegetative cells; in mating; ascospore chitosan layer	Transcription activated by Calcofluor white and in sporulation	48, 50, 82, 84, 333, 336, 372, 380, 393, 427
CHS4[a] (*CSD4*)	No	2L	Required for CSIII	As above but not the ascospore chitosan layer	Posttranslational activator of chitin synthase III?	48, 49, 372
CHS5[a]	No	12R	Required for CSIII	As above but not the ascospore chitosan layer	Essential for mating (in homozygosis)	372, 380
SHC1	No	5R	Related to CSD4	Ascospore chitosan layer?	Transcription activated in sporulation?	49
CSD1	?	?	Required for UDP-GlcNac synthesis?	?		48, 49
CHS6[a] (*CSD3*)	No	10L	?	?		48, 49

[a] *CAL1*, *CAL2*, and *CAL3* names have been withdrawn; the new names are *CHS3*, *CHS4*, and *CHS5* (standing for chitin synthesis), respectively (59).

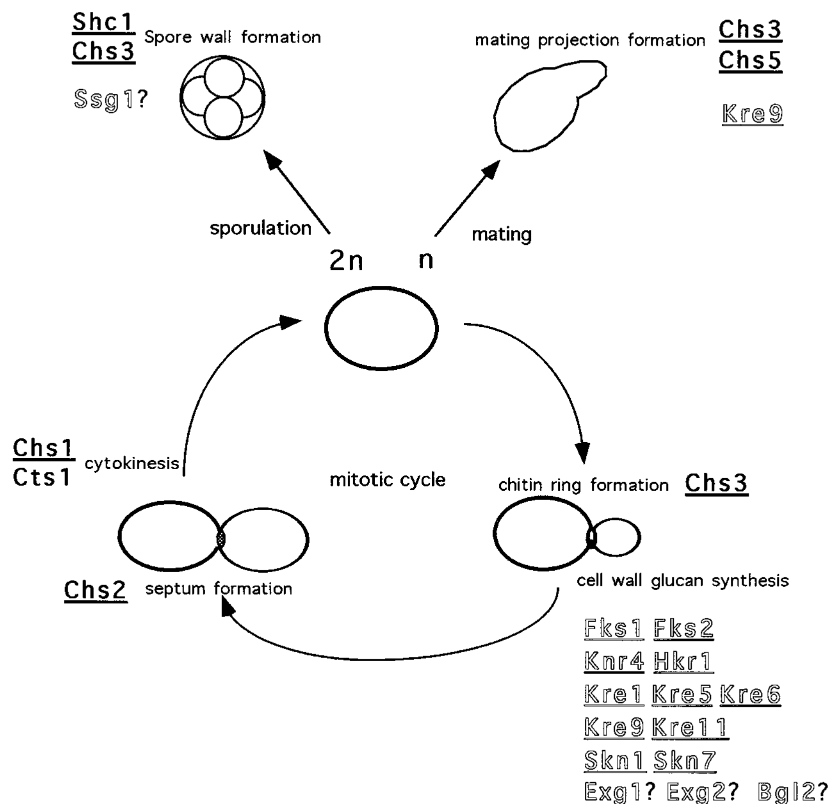


FIG. 3. Gene products related to cell wall enzymatic dynamics at different stages of the life cycle. Underlined genes are related to synthetic pathways; the nonunderlined ones correspond to hydrolases. Outlined genes are related to β-glucan dynamics, whereas nonoutlined ones are those related to chitin.

distributed on the yeast plasma membrane in the zymogenic form (128, 130), although a complementary location in vesicles (called chitosomes) has also been proposed (250). Solubilization by digitonin still elicits zymogenic activity, which in vitro requires controlled proteolytic pretreatment for its activation (129). Extensive purification by entrapping the enzyme in its own product has been achieved (223).

The isolation of a mutant (*chs1*) apparently lacking CS in vitro but showing a normal growth rate and normal chitin contents led to the cloning of the corresponding *CHS1* wild-type allele (51). It was shown that the cloned gene was the structural gene for CSI and that its disruption neither was lethal nor affected chitin synthesis in vivo (51). This suggested the requirement of an additional CS activity in *S. cerevisiae* (see below).

When a *CHS1* disruptant grows in minimal media (more accurately, in poorly buffered media), small refractile buds are produced (51). The refractility of the buds is due to a loss of permeability. Under electron microscopy, bud lysis seems to be a consequence of the appearance of a hole in the cell wall, located in the center of the birth scar of the daughter cell at the time of cell separation (65). The defect can be prevented by buffering the growth medium or by adding sorbitol as an osmotic stabilizer. A chitinase (see below) located in the periplasmic space, with a very acidic optimal pH (96, 132), is most probably responsible for cell separation owing to partial degradation of the chitin of the primary septum (247, 376) and may also be responsible for lysis in *chs1* strains. Reducing the chitinase activity level by increasing the pH value of the medium or by using specific inhibitors—such as allosamidin or demethylallosamidin—minimizes lysis in *chs1* mutants (65). Furthermore, double mutants carrying a disruption of the chitinase structural gene (*CTS1* [see below]) and the *chs1* mutation are not always lysed at acidic pH values (67). The conclusion from these findings is that CSI prevents the lysis caused by excessive chitinase activity at low pH and that CSI must therefore exert a repair function at the end of cytokinesis, counterbalancing the physiological lytic effect of the chitinase (Fig. 3). However, some *chs1* strains do not show lysis. Genetic analysis has shown that the nonlytic phenotype behaves as a recessive character. This suggests that in addition to *CTS1*, the product of at least another gene, *SCS1* (standing for suppressor of chitin synthesis), is required for lysis (67).

Mating and shmoo formation are apparently unaffected in *chs1* disruptants (51). By contrast, it is known that the synthesis of chitin in these events is due to CSIII activity (see below) (427). However, the possibility that *CHS1* plays a subtle repairing role during mating (as happens during the mitotic cycle) has not been discarded. In this sense, mating between complementary strains carrying both *CHS1* and *CHS3* disruptions merits attention. Also, sporulation and synthesis of the spore chitosan layer are not affected in homozygous *chs1* mutants (51, 336).

Chitin synthase II: genes involved and function. CSII can be readily measured in a *chs1* mutant background. Like CSI, CSII is located on the plasma membrane and can be activated in vitro by treatment with proteases (385). Two major differences allow a distinction between CSI and CSII as measured in vitro: (i) Co^{2+} is the best metal stimulator for CSII, whereas it inhibits CSI; and (ii) the optimum pH for CSII (pH 7.5 to 8) is more alkaline than for CSI (pH 6.5) (385).

CHS2, the gene that codes for CSII, was cloned by detection

of its overexpression in vitro, on a *chs1* background (401). As with *CHS1*, the transformation of *Schizosaccharomyces pombe* (used as a heterologous system) with a plasmid containing the *CHS2* gene led to the expression of a CS with characteristics similar to those of the *S. cerevisiae* CS (401). However, the use of *S. pombe* in these experiments as a nonchitinous yeast should probably be reconsidered, since some chitin and also some CS have been detected in *S. pombe*, as well as a DNA sequence with homology to the budding yeast *CHS* genes (38, 400).

CHS2 was originally described as an essential gene (401). However, it was later shown that in certain strains and under specific nutritional conditions (namely, the use of synthetic medium or rich medium with glycerol as the carbon source), *chs2* spores are viable (50). Hence, the *CHS2* gene was eventually shown to be nonessential. Remarkably, a disruptant of both the *CHS1* and *CHS2* genes had no in vivo defect on chitin synthesis. Moreover, it had a measurable CS in vitro (50). A third CS would be responsible for such phenomena.

Initial observations indicated that no defined primary septa could be observed in strains lacking a functional *CHS2* gene (401). Instead, thick amorphous septa are formed at the neck region between mother and daughter cells (401) and chitin is overproduced up to twofold compared with the wild-type level (50). The cells are abnormally large and aggregate in clumps that are not easily broken by sonication (393). Under normal conditions, nuclear segregation is accomplished before septum formation; however, multinucleate cells can be seen in *chs2* mutant cultures (49, 50). All these morphological alterations persist in the presence of a concurrent *chs1* mutation. The conclusion is that the *CHS2* gene must be involved in primary septum formation and, perhaps, in some related final step of cytokinesis (Fig. 3).

Chitin synthase III: genes involved and function. In retrospect, it is now clear that CSIII was previously detected in a *chs1* background (333). Ni^{2+} is a powerful inhibitor of CSI and CSII but has very little effect on CSIII, especially in the presence of Co^{2+} (83). The use of different divalent cations and pHs enables the specific determination of three *S. cerevisiae* CSs (83). CSIII was first considered nonzymogenic (50, 393). However, recent results have shown that treatment of cell extracts with detergents leads to a considerable reduction in CSIII that can be not only restored but also increased (10-fold) after subsequent incubation with a protease in the presence of substrate (84). Therefore, CSIII also behaves, at least in vitro, as a zymogen.

The first approach to the isolation of genes involved in chitin synthesis (CSIII related) took advantage of the fact that Calcofluor white interferes with chitin synthesis (370); indeed, under appropriate circumstances, it is toxic for yeast cells (371). A correlation was established between the presence/absence of chitin and sensitivity/resistance to the fluorochrome, and therefore a search for Calcofluor white-resistant mutants (*cal* mutants) was undertaken (372). To summarize, the main characteristics of these mutants were (i) resistance to Calcofluor white, (ii) a considerable reduction in the amount of cell wall chitin both in vegetative cells and after α-factor treatment, and (iii) CSI and CSII levels similar to those of the wild type. The second approach consisted of the isolation of mutants defective in the incorporation of tritiated glucosamine into chitin in vivo (chitin synthesis defective mutants) (*csd1*, *csd2*, *csd3*, and *csd4*) (48).

Complementation analysis performed by C. E. Bulawa indicated the existence of three different complementation groups for Calcofluor white resistance: *cal1*, *cal2*, and *cal3* (with *csd2* and *csd4* being allelic to *cal1* and *cal2*, respectively) (48). Since the *cal* denomination had previously been used for a different purpose (329), it seemed appropriate to withdraw it. Mutants defective in the chitosan and the dityrosine-rich layers of the spore wall (*dit101*) (336) or resistant to exogenous *Kluyveromyces lactis* killer toxin (*kti2*) (52, 419) also have mutations that map in the *csd2* locus.

According to a recent proposal for nomenclature (59), largely endorsed by the authors involved in the discovery of genes related to chitin, the *CAL1/CSD2/DIT101/KTI2*, *CAL2/CSD4*, and *CAL3* genes would now become *CHS3*, *CHS4*, and *CHS5*, respectively, whereas *CSD3* would become *CHS6* (all of them standing for chitin synthesis-related). Among all the *chs* mutants, only *chs3*, *chs4*, and *chs5* are defective in CSIII in vitro (48, 49, 427). It may therefore be concluded that this activity requires at least the products of these three genes.

The *CHS3* gene was cloned by complementing the Calcofluor white resistance phenotype. In parallel, cell wall chitin and CSIII wild-type levels were also restored (427). Disruption of *CHS3* is not lethal and promotes a phenotype identical to that of the original *chs3* mutant. Cloning of *CHS3* has also been described by Bulawa (48). That report extended the *CHS3* sequence 198 bp more at the amino-terminal region than did previous reports; this difference is probably due to a sequencing error in the first reported sequence (427). The predicted protein sequence shows a reduced but statistically significant homology with those of *CHS1* and *CHS2*, allowing us to consider *CHS3* to be the structural gene for CSIII or a subunit thereof. Nevertheless, overexpression of *CHS3* does not lead to overproduction of CSIII, and transformation of *S. pombe* with *CHS3* does not raise CSIII levels either (427). These results indicate that *CHS4* or *CHS5* or both are probably limiting factors for CSIII activity.

The *CHS4/CSD4* sequence shows no homology to CS genes, and its disruption is not lethal (49). *CHS4* overexpression increases CSIII activity severalfold. However, simultaneous overproduction of *CHS3* and *CHS4* does not further increase CSIII (49).

The *CHS5* gene has been cloned by complementing the Calcofluor white resistance phenotype in a *chs5* background (380). Defects in the amount of cell wall chitin and CSIII activity were also complemented. The predicted sequence of Chs5p has a 19% identity and a 70% similarity (over a region of 401 residues) with that of the protein H of neurofilaments (254, 380). It shows two structural peculiarities: a string of 10 heptameric repeats, and a C-terminal tail rich in lysines. Disruption of the *CHS5* gene is not lethal and mimics all the phenotypes ascribed to the *chs5* mutant. Unlike *CHS4*, *CHS5* overexpression does not increase CSIII levels (380).

chs3, *chs4*, and *chs5* mutants, defective in CSIII, have reduced amounts of chitin in their cell walls (48, 372). Therefore, CSIII must be responsible for the synthesis of most of the chitin in the vegetative cell. Chitin deposited as a ring at the neck region between mother and daughter cells is clearly absent in *chs3* mutant cells (393). The residual amount of chitin uniformly dispersed in the lateral wall of mother cells, which is detectable by wheat germ agglutinin-fluorescein isothiocyanate complex staining, is also missing in *chs3* mutants (372). In fact, the increased deposition of chitin on the lateral wall of *cdc24* or *cdc3* mutants (see below) (362, 404) is abolished in *cdc24 chs3* or *cdc3 chs3* double mutants (393). Nevertheless, unlike *chs2* cells, *chs3* mutant cells keep the typical trilamellar structure of the septa. However, these are convex rather than flat as in wild-type cells, and, as a consequence, the bud scars are also convex and distended, giving the cells a "bumpy" appearance (393). By specific staining with wheat germ agglutinin–colloidal-gold complexes, chitin in *chs3* mutants can be clearly ob-

served to be present only on the primary septum, thus providing further evidence for the role of Chs2p as being responsible for chitin synthesis at this point (393) (see above).

Additional relevant phenotypes ascribed to *chs3* mutants merit comment. (i) The alkali-soluble β-glucan cell wall fraction from *chs3* mutants increases at the expense of the alkali-insoluble one (372); this result is consistent with the proposal that the insolubility of β-glucan in alkali results from its attachment to chitin (13, 299, 399) and favors the recently confirmed hypothesis (181, 234) suggesting the existence of β-glucan–chitin complexes. (ii) *chs3* mutants are resistant to exogenous *K. lactis* toxin, in agreement with the idea that chitin is the cell wall receptor for this toxin (53, 419). (iii) There is no induction of chitin synthesis after pheromone treatment in *chs3* mutants; however, although the shmoo displays a different morphology, mating is not impaired even when both counterparts (**a** and α) are *CHS3* deficient (372). (iv) Sporulation in *chs3* homozygous diploids is unaffected, but the chitosan layer in the ascospore is missing (336). All these results clearly suggest that CSIII is in charge of the bulk of chitin synthesis not only during the mitotic cycle but also during mating and sporulation (Fig. 4), although its function is dispensable for the completion of these processes.

CHS4 and *CHS5* are limiting factors for CSIII and can be considered either modulators or subunits of such activity. However, they are not completely indispensable for CSIII. *chs4* and *chs5* mutant cells, unlike *chs3*, have a low although detectable level of chitin in the lateral wall and in shmoos (372). *CHS4* is not required for chitosan synthesis during sporulation. Rather, it seems that *SHC1* (see below) may be its substitute in this circumstance (cited as unpublished results in reference 49). Neither sporulation nor synthesis of the ascospore chitosan layer is significantly affected in *chs5* homozygous diploids (380).

CHS4 does not seem to be a transcriptional activator of *CHS3* (49). However, overexpression of *CHS4* or the addition of extra *CHS4* gene product to lysates from wild-type cells does stimulate CSIII activity (49). In addition, *chs4* but not *chs3* or *chs5* mutants are able to produce trypsin-elicited CSIII in vitro (84). Hence, *CHS3* and *CHS5* gene products appear to be necessary components for zymogenic CSIII, whereas Chs4p may be a posttranslational modulator of this activity.

As deduced from its sequence, the structure of the *CHS5* gene product favors its consideration as a protein that may form oligomers or some sort of filaments with structural function (380). *CHS5* does not seem to be a transcriptional activator of *CHS3* (106). Transcription of *CHS5* in synchronous cultures has not been analyzed, but *CHS5* mRNA levels do not vary from the logarithmic to the stationary phase, neither after α-factor treatment nor during sporulation (380). However, *chs5* disruptants show a very early defect in mating and display a phenotype quite similar to that observed in typical mating-defective strains, such as *fus1 fus2* double mutants (380). Thus, the *CHS5* gene seems to exert two functions: a dispensable one as regards chitin synthesis (CSIII) and another one that is very important for mating.

It is difficult to envisage a role for the *CHS4* and *CHS5* gene products with currently available information. They may serve as a structural component of a delivering system involved in polarity processes. Furthermore, it is well known that mutants defective in actin (319), actin-capping protein (5), profilin (174), verprolin (119), other proteins related to actin function, or the putative components of the neck filaments (87, 403) (see below) display an altered pattern of chitin deposition. It is therefore tempting to speculate that the *CHS4* and *CHS5* gene products would link chitin synthesis with morphogenetic events in which cytoskeletal or regulatory components were involved.

Structure-function relationships in chitin synthase genes. Comparison of the predicted amino acid sequences from *CHS1* and *CHS2* points to a close relationship between them (40% identity in the last 750 amino acids). They also share a reasonable homology with *CHS3*, mainly restricted to the C-terminal third of the sequences. The hydropathic profiles of *CHS1* and *CHS2* gene products are quite similar and differ from that of *CHS3*. The amino-terminal region shows no homology among the three sequences, and a considerable portion of it seems to be nonessential. The *CHS1* N-terminal region nonhomologous to *CHS2* (389 amino acids) can be deleted without loss of activity or function (66), and deletion of the first 221 amino acids in *CHS2* does not affect the enzymatic activity (66). By contrast, the C-terminal region appears to be essential, since removal of the last 75 amino acids in *CHS2* or 43 amino acids in *CHS3* leads to loss of the corresponding activity and function (66, 106, 393).

The spatial alignments of *CHS1*, *CHS2*, and *CHS3*, in comparison with already crystallized related enzymes, identified a putative region for nucleotide binding (157, 418). According to this, Lys-1125 or Lys-1126 *CHS3* residues would be essential. However, in vitro mutagenesis experiments were performed with negative results (106). More recently, hydrophobic cluster analysis of a series of β-glycosyl transferases (including Chs1p) has allowed the localization of two domains presumably involved in the formation of the β-linkage in processive transferases that use nucleoside diphosphate sugars as donors (384). In fact, the essentiality of several residues located in these domains in a presumptive catalytic site of Chs2p has been demonstrated very recently (309a).

The alignment of *CHS1*, *CHS2*, and a CS from *Candida albicans* (11) sequences allowed the identification of conserved sequences that were then used to design PCR primers and the amplification of fragments from several real or putative CSs from different yeasts and fungi (38, 454). In this way, CSs from *C. albicans* (81), *Neurospora crassa* (453, 454), and *Ustilago maydis* (167) have been found. Moreover, the alignment of *S. cerevisiae CHS1*, *CHS2*, and *CHS3* sequences defines different PCR primers, opening the possibility of searching for new *CHS3*-type sequences among yeasts and fungi. Indeed, using this approach, a third chitin synthase gene from *C. albicans*, showing a sequence very similar to that of *CHS3*, has been isolated (412).

Other genes involved in chitin synthesis. The *CSD1* gene is defined by *csd1-1*, a temperature-sensitive mutant in which both growth and chitin synthesis are blocked at the nonpermissive temperature (48). It has been proposed that *CSD1* may be required for UDP-GlcNAc synthesis (49).

CHS6/*CSD3* is another gene related to chitin synthesis, but it is not required for CSIII activity (48). It encodes a hydrophilic protein that shows no similarity to any known protein. Disruption of this gene has shown that it is not essential and therefore is probably not involved in UDP-GlcNAc biosynthesis.

The *SHC1* gene was found in protein database searches for sequences similar to *CHS4* (49% identity over 220 amino acids) (*SHC* stands for sporulation-specific homolog of *CHS4*/*CSD4*) (49). A strain disrupted in the *SHC1* gene synthesizes normal amounts of chitin during vegetative growth and shmoo formation. *SHC1* mRNA is undetectable during exponential growth, but its levels rise dramatically during sporulation. It has therefore been proposed that *SHC1* is required for chitin synthesis during sporulation (49).

Additional mutants resistant to Calcofluor white have been

obtained, but all of them are sterile (372). Some of them show a defect in chitin synthesis similar to that observed in the *chs3* mutant, while chitin levels in others are comparable to those in wild-type strains. Recently, a new Calcofluor white-resistant locus other than those described up to date has been obtained (423).

Regulation of chitin synthesis. The existence of three different CSs with defined roles throughout the yeast life cycle suggests the need for specific temporal and spatial mechanisms involved in the regulation of their function. Temporal regulation can be achieved by the synthesis of the enzyme when required followed by degradation once its function has been accomplished. Another alternative would consist of rounds of activation and inactivation of a latent enzyme at the appropriate time. Spatial regulation requires either a mechanism responsible for accurate location of the active synthase or a strictly located activation of a randomly dispersed enzyme at the actual site where chitin synthesis is required. CSI and CSII appear to be necessary only at a single moment and at a precise site during the mitotic cycle (Fig. 3). By contrast, chitin synthesis by CSIII occurs at several places and moments during the yeast life cycle; therefore, it seems reasonable to assume that its regulation may be more complex.

The *GFA1/GCN1* gene codes for L-glutamine:fructose-6-phosphate amidotransferase (442, 447), the first enzyme in the hexosamine biosynthetic pathway. Both the transcription of this gene and its activity levels have been shown to be increased by α-factor treatment (442). In the light of these findings, substrate availability may play a role in the regulation of chitin synthesis during mating. A similar suggestion has recently been made for *Blastocladiella emersonii* cell wall biogenesis (102).

In principle, synthase zymogenicity reflects an in vitro requirement for proteolytic activation; nevertheless, proteinase B, previously considered an in vitro activating factor for CSI (64), is not required for CSI function in vivo (402). Despite this, physiological activation of some CSs must be accomplished either by other proteases or by some other unknown nonproteolytic procedure. As mentioned above, *CHS4* and *SHC1* may act as hypothetical posttranslational regulators of CSIII in the vegetative cycle and mating or in sporulation, respectively.

As assayed after proteolytic activation, CSI remains constant throughout the cell cycle (82) but the transcript level of *CHS1* appears to be regulated through the cell cycle and peaks at the time at which CSI is presumably required (336). Expression of *CHS1* under the control of a *GAL1* promoter has been studied. After the cells were shifted from galactose to glucose, *CHS1* transcription was turned off and the decay in mRNA and enzyme activity was monitored. *CHS1* mRNA disappeared rapidly, whereas the activity remained fairly stable (82). The transcription of *CHS1* and the synthesis of CSI are clearly stimulated by mating pheromones (9, 388). The *CHS1* sequence contains four copies of the specific pheromone-responsive element at the 5′ noncoding region (9). In spite of these results, *CHS1* disruptants apparently have no obvious defect either in shmoo formation or in mating. Therefore, the contribution of CSI to the synthesis of chitin in these morphogenetic events appears to be unsubstantial. *CHS1* transcription is not induced in sporulation (380). In conclusion, the possibilities for transcriptional and/or posttranslational regulation of CSI remain open.

CSII is also a zymogenic activity, although so far no physiological activator has been described. Transcription of *CHS2* seems to be cell cycle regulated and peaks around the time of formation of the primary septum (336). The level of CSII activity, measured after trypsin treatment, also shows a peak around the time of septation (82). By contrast, in the presence of sexual pheromone or during sporulation, *CHS2* transcription is practically abolished (82, 336). In a set of experiments similar to those mentioned above for *CHS1*, it was observed that the levels of CSII activity and *CHS2* mRNA decay rather fast (82). From this, it can be concluded that *CHS2* may be regulated transcriptionally, although strictly speaking, posttranslational regulation should not be ruled out. Indeed, four consensus sequences for Cdc28 kinase phosphorylation are localized at the beginning of the *CHS2* sequence.

Much remains unknown about CSIII regulation, even though several genes involved in such activity have been already cloned (see above). Some fluctuations in *CHS3* mRNA levels have been observed throughout the cell cycle (336). There is no information about CSIII variations along the cell cycle. The results on the decay of CSIII activity and *CHS3* mRNA levels after turning off *CHS3* overexpression in experiments similar to those described previously for *CHS1* or *CHS2* are difficult to interpret (82).

Calcofluor white presumably alters chitin synthesis by preventing the formation of chitin fibrils and by interfering with the process of crystallization. Before growth arrest in the presence of this chemical, chitin synthesis is activated at the level of the septa (370). Paradoxically, Calcofluor white is an inhibitor of CSs in vitro (370), so its in vivo effect must be caused without its entering the cell (370, 371). Activation of chitin synthesis by Calcofluor white is mediated by (i) an increase in CS levels (presumably CSIII), which is abolished by cycloheximide (371), and (ii) a specific activation of *CHS3* transcription (the transcription of *CHS1*, *CHS2* or *CHS5* genes is unaffected) (380). This activation is apparently not mediated either by the α-factor or by *PKC1*-dependent signal transduction pathways (see below) (106).

CSIII and *CHS3* mRNA levels remain constant during pheromone treatment. The *CHS3* gene has two consensus sequences for α-factor activation in its 5′ noncoding region, although one of them may not be functional (49). No measurement of CSIII during sporulation has been reported; however, in contrast to other findings (336), *CHS3* mRNA levels rise drastically after 10 h under sporulating conditions (82), at the time when later genes (such as those involved in spore wall synthesis) are expressed. The overall conclusion is that CSIII appears to be regulated transcriptionally and posttranslationally.

All CSs are devoid of the conventional amino-terminal signal sequence for secretion. Nevertheless, the delivery of these enzymes to the plasma membrane—the place where chitin microfibrils are vectorially extruded to the cell wall—requires a transport system. Chitosomes may constitute this kind of element involved in the spatial regulation of chitin synthesis (21). It has been suggested that *CSD3* may play a role in the spatial regulation of chitin synthesis (49). As mentioned above, *CHS5* may also be involved in this process.

Mutants affected in the formation of the 10-nm microfilament ring at the neck region in the submembrane between mother and daughter cells (*cdc3*, *cdc10*, *cdc11*, and *cdc12*) (see below) are defective in cytokinesis, and their morphologies are altered (56, 87, 182). It is assumed that these microfilaments would play diverse roles along the cell cycle (see below). Some arguments relate the putative neck filament constituents to the spatial regulation of chitin synthesis: (i) the disassembly of the neck filament ring of these mutants runs parallel to a disgregation of the chitin ring in the wall above this structure (87, 362, 403), and (ii) mutants with mutations in the genes assumed to code for the components of this ring show a bumpy

phenotype reminiscent of that previously described for *chs3* mutants (87). Thus, a functional connection between those genes and CS is likely.

Chitin synthesis as a target for antifungal drugs. Chitin is a fungal cell wall structural polysaccharide not found in mammals. Inhibitors of chitin synthesis are very specific agents against pathogenic fungi but are nontoxic to the host. This approach presupposes that the inhibition of chitin synthesis is lethal for the fungal cell. Single or double mutants lacking CSI and CSII or CSI and CSIII are viable. Searches for mutants defective in all three activities through genetic crosses between single or double mutants have been unfruitful, indicating that they may be nonviable (49, 393). In a different approach, a *chs1 chs2 chs3* triple mutant carrying a plasmid containing the *CHS2* gene under the control of *GAL1* promoter was constructed (393). The mutant grows on galactose medium, but when it is transferred to glucose, *CHS2* is turned off and the mutant cells finally die; the lethality is not alleviated by the presence of the *CHS1* gene (393). Thus, chitin is essential for yeasts and can therefore be considered a useful target in the search for antifungal agents.

The use of chitin synthesis inhibitors both in vivo and in vitro has been very useful in the study of the enzymology of the system, and for more details the reader is referred to other reviews (105, 163, 169). In short, polyoxins and nikkomycins are very powerful competitive inhibitors of CSs, since they are structural analogs of the substrate. However, because of the existence of several CSs with different degrees of susceptibility to these inhibitors (58, 161), caution should be exercised at the time of choosing the in vitro assay when screening for this kind of antifungal agent. Two main conclusions can be drawn from experiments on chitin synthesis inhibition by polyoxins: (i) these inhibitors exert their action as expected, i.e. at the septal region and at the time of cytokinesis; and (ii) unfortunately, in many cases the in vivo MICs of these inhibitors are much higher than the corresponding inhibitory constants as determined in vitro. It appears that either inability to permeate the fungal plasma membrane or instability inside the fungal cell is the major remaining problem in the use of these drugs as antifungal agents.

β-Glucan

β-Glucan is the most abundant component of the yeast cell wall, and, unlike chitin, it is uniformly distributed around the cell. The major structural component is a large linear 1,3-β-glucan polymer branched with some 1,6-β residues (238, 239, 287). There is another minor, highly branched component, 1,6-β-glucan with occasional 1,3-β linkages and 1,6-β-linked side chains (286). For more information about the structure of yeast β-glucan, the reader is referred to recent reviews (142, 375) and to the section on cell wall molecular architecture, above.

Enzymology of β-glucan synthesis. Yeast cell wall β-glucan is the generic name used to designate the complex structure that consists of a family of polysaccharides containing chains of 1,3-β- and 1,6-β-linked glucose units involved in the support and maintenance of cell wall integrity. Nothing is known about the biochemistry of the formation of 1,6-β bonds, but valuable information has been gathered on the biosynthesis of 1,3-β-glucan by cell extracts from *S. cerevisiae*. After very preliminary trials (15, 272, 391), in vitro biosynthesis of this polymer was efficiently achieved (394). The sugar donor was found to be UDP-glucose, and the enzyme preparation required the presence of EDTA, GTP, or ATP and other components for maximal activity. The reaction product was characterized as a 1,3-β-linked linear glucan, and the activity was found in the plasma membrane fraction (394). Further studies clearly showed that nucleoside triphosphates, especially GTP and its analogs, acted as powerful stimulators of the synthase (318, 395). These results were extended to other yeasts and fungi belonging to different taxonomic groups (344, 359, 417, 435), leading to the proposal that stimulation of synthase activity by nucleotides might be a general regulatory mechanism for 1,3-β-glucan synthesis.

Dissociation of the activity into two proteinaceous components (222) by extraction of the membranes with NaCl and detergents disclosed the existence of a membrane-bound fraction, which seemed to contain the catalytic center of the enzyme, and a detergent-solubilized GTP-binding component that may play an important role in the regulation of 1,3-β-glucan biosynthesis (63). So far, biochemical characterization of the system has been precluded by the great difficulty in purifying the components to homogeneity. However, a 20-kDa GTP-binding component has recently been purified (300). This preparation no longer required GTP for activity, although incubation with another fraction obtained in the purification procedure resulted in the hydrolysis of the bound GTP to GDP and a concomitant requirement of GTP for activity (300). Photoaffinity labelling with UDP-glucose analogs should allow the detection of the UDP-glucose-binding component of the system, as has been recently described for *N. crassa* (134). The use of cell extracts from systems in which 1,3-β-glucan synthesis is especially activated, i.e., regenerating protoplasts in the presence of either Calcofluor white or Congo red (370), might facilitate this search.

Genes involved in 1,3-β-glucan synthesis. To gain further insight into the physiologically relevant mechanisms involved in yeast 1,3-β-glucan synthesis, several genetic approaches have recently been developed. Different strategies have been used to isolate mutants and/or the genes involved, to different extents, in 1,3-β-glucan synthesis. A list of genes involved in β-glucan synthesis is given in Table 2.

(i) Genes defined by resistance to several 1,3-β-glucan synthesis inhibitors or by hypersensitivity to Calcofluor white. One approach addressed the isolation of mutants resistant to antifungal agents that act on 1,3-β-glucan synthesis, such as papulacandin B, *pbr1* (papulacandin B resistance) (71); aculeacin A (149); or echinocandin, *etg1* (echinocandin target gene) (121, 133). Papulacandin B was one of the first antibiotics reported to inhibit yeast growth by specifically interfering both in vivo and in vitro with 1,3-β-glucan synthesis in yeasts and filamentous fungi (14, 71, 344, 435). *ETG1* (120) and *PBR1* (71) genes have been independently cloned and characterized by these techniques.

A broader approach for isolating cell wall-defective mutants, by screening for hypersensitivity to Calcofluor white, has recently been described (353). On the basis of the known toxicity of Calcofluor white because of its interference in cell wall assembly (370), it was assumed that mutants affected in functions involved in the preservation of cell wall integrity might be more susceptible to this agent. In this search, 53 complementation groups were reported; among them, the *cwh53-1* mutant and others displayed low glucose levels in their cell walls, suggesting a defect in β-glucan synthesis (353). *CWH53*, the gene complementing the *cwh53-1* mutation, has been cloned and characterized (352).

By sequence comparison, *CWH53*, *ETG1*, and *PBR1* define the same gene. In addition, *CWH53* is identical to *CND1*, a gene whose mutation is synthetically lethal with calcineurin (a Ca^{2+}/calmodulin-dependent protein phosphatase) mutations (352). Furthermore, *ETG1* is identical to *FKS1* (120, 135), a

TABLE 2. Genes involved in cell wall β-glucan synthesis

Gene (synonym)	Essential	Chromosomal location	Selection marker	Proposed function	Reference(s)
CWH53[a] (*FKS1*, *ETG1*, *PBR1*, *CND1*)	No	12R	Hypersensitive to Calcofluor white or FK506; resistance to echinocandin or papulacandin B; synthetic lethality with calcineurin mutants	1,3-β-Glucan synthase I component	71, 120, 121, 135, 352
FKS2	No	?	*FKS1* homolog	1,3-β-Glucan synthase II component	151
KNR4 (*SMI1*)	No	7R	*H. mrakii* killer toxin resistance	1,3-β-Glucan synthesis regulator	197
HKR1	Yes	?	*H. mrakii* killer toxin resistance by overexpression	1,3-β-D-Glucan synthesis regulator	248
KRE1	No	14	K1 killer toxin resistance	1,6-β-D-Glucan synthesis	37
KRE5	No	15R	K1 killer toxin resistance	1,6-β-D-Glucan synthesis	295
KRE6	No	16	K1 killer toxin resistance	1,6-β-D-Glucan synthesis	367–369
KRE9	No	10L	K1 killer toxin resistance	1,6-β-D-Glucan synthesis	44
KRE11	No	7R	K1 killer toxin resistance	1,6-β-D-Glucan synthesis	45
SKN1	No	7	K1 killer toxin resistance	1,6-β-D-Glucan synthesis	368, 369

[a] In addition to *cwh53*, several other *cwh* mutants (including *cwh52*) have a low glucose cell wall content; it has been proposed that the decrease in the amount of glucose indicates a defect in 1,3-β-glucan synthesis (353).

gene cloned by complementation of a mutant hypersensitive to the immunosuppressants FK506 and cyclosporin A, which are calcineurin inhibitors (151).

The *CWH53/ETG1/FKS1/PBR1/CND1* gene codes for a fairly large polypeptide (215 kDa) that was predicted to be an integral membrane protein with 16 potential transmembrane domains and a highly charged N terminus. The gene is not essential for vegetative growth (71, 120), although the generation time of the disruptant doubles that of the isogenic wild-type strain and the morphology of disruptant cells is altered (71). The fact that addition of sorbitol to the medium corrects these differences is an indication that the disruptant has an osmotically fragile cell wall. Indeed, cell wall fractionation analysis indicates that the levels of alkali-insoluble and alkali-soluble cell wall fractions of a *pbr1* disruptant are clearly reduced compared with those of the isogenic wild-type (71). Furthermore, 1,3-β-glucan synthase activity from disruptant extracts is reduced to at least 50% of that of the wild-type (71, 120) while the introduction of low-copy-number or multicopy plasmids carrying *PBR1* restores the synthase activity to control levels (71).

etg1-1 and *pbr1-1* mutations result in echinocandin- and papulacandin B-resistant 1,3-β-glucan synthases, respectively. No other kinetic properties of the enzymes are affected by the mutations (71, 121). From these observations, it can be concluded that these mutations specifically affect the interaction between the drug and the enzyme without interfering with UDP-glucose binding. In the case of *etg1-1*, it has also been shown that the resistant phenotype can be ascribed to the catalytic subunit of the synthase (121).

In summary, the *CWH53/ETG1/FKS1/PBR1/CND1* locus seems to be responsible for papulacandin B and echinocandin sensitivity as well as for normal 1,3-β-glucan content and synthase levels. Thus, the product of this gene can be considered a structural component of the 1,3-β-glucan synthase complex. However, it should be stressed that no UDP-glucose-binding consensus sequence (RXGG) (137, 285) has been found in the encoded polypeptide; this may be interpreted as an indication that the gene product might not be the catalytic subunit but, rather, another component of the 1,3-β-glucan synthase complex.

The residual synthase activity present in the disruptant, together with the fact that the disruption is not lethal and the presence of a second sequence that cross-hybridizes with either *PBR1* or *FKS1/ETG1* probes under low-stringency conditions (71, 120), pointed to the existence of a second homolog redundant gene. The papulacandin B sensitivity of the *pbr1* disrupted strain (71) and the higher sensitivity to echinocandin of the residual synthase activity in *fks*Δ*1* or *fks1-1* strains compared with those in the wild type (120) also supported this hypothesis. Indeed, an *FKS1/ETG1* highly homologous gene, *FKS2*, has been described, and the simultaneous disruption of both genes appears to be lethal (151).

FKS2 transcription is dependent on calcineurin and is strongly inhibited by FK506 and cyclosporin A (cited in reference 352). This explains why *fks1* mutants are hypersensitive to these immunosuppressors. In the presence of FK506 or in calcineurin mutants, *FKS2* is not transcribed and 1,3-β-glucan synthesis depends only on *FKS1*. Since 1,3-β-glucan synthesis is essential for the yeast cell, mutations in *FKS1* in the presence of FK506 or cyclosporin A or in a calcineurin mutant are lethal (cited in reference 352). Regulation of *FKS2* by calcineurin suggests phosphorylation/dephosphorylation as a regulatory mechanism for 1,3-β-glucan synthesis and involvement of Ca^{2+} on it. *CWH53/FKS1* transcription also appears to be cell cycle regulated, showing a maximum in the late G_1/S phase (352).

S. pombe papulacandin B resistance mutants (*pbr*) have been also isolated, and all the mutants define a single complementation group (*pbr1*) (71), but the heterologous *S. cerevisiae PBR1* gene does not complement their defects, in spite of the apparently similar behavior of the *S. cerevisiae* and *S. pombe pbr1* mutants (71).

GNS1 is a novel gene which has been cloned by complementation of a mutation conferring resistance to another echinocandin analog (133a). *gns1* mutant strains exhibit a pleiotropic phenotype including a drastic reduction in 1,3-β-glucan synthase specific activity as measured in vitro. *GNS1* is nonessential, and the encoded product is predicted to be an integral membrane protein with five transmembrane helices (133a), which may constitute either a subunit or a regulator of the 1,3-β-glucan synthase.

A different approach to the search for β-glucan synthesis-related genes consists of the isolation of mutants resistant to the K9 killer toxin from *Hansenula mrakii*. This toxin has been

described as a selective inhibitor of β-glucan synthesis in *S. cerevisiae* (450). *knr* (for killer nine resistance) mutants fell into nine complementation groups (197). The *KNR4* gene has been cloned and analyzed (197). It codes for a protein showing 100% identity to the nuclear protein coded by the *SMI1* gene, whose disruption produces a temperature-sensitive cell cycle S-phase arrest (141). The *KNR4* product displays a hydrophilic profile and contains several proline, glutamate, serine, threonine (PEST)-rich regions, usually found in rapidly degraded proteins, and several putative sites for phosphorylation by protein kinase C and casein kinase II. Disruption of *KNR4* is not lethal; however, it promotes a considerable reduction in both 1,3-β-glucan synthase activity and 1,3-β-glucan content in the cell wall (197) but an increase in the chitin content (198). It is suggested that *KNR4* may encode a protein that coordinately regulates cell wall synthesis, either directly or by interacting with another regulator. Cloning of the *gs-1* gene from *N. crassa* by functional complementation of the cell wall defect of a 1,3-β-glucan synthase-deficient mutant has been reported recently (134). The predicted encoded protein is partially homologous to Knr4p/Smi1p.

Overriding the effect of *H. mrakii* K9 killer toxin by overexpression of wild-type genes has allowed the isolation and characterization of *HKR1* (for *H. mrakii* killer toxin resistant) (248), an essential gene that encodes a presumptive calcium-binding glycosylated type I membrane protein. Overexpression of the C-terminal half of Hkr1p rendered the cells more resistant to the toxin and increased the alkali-insoluble β-glucan content but did not increase the in vitro measurable level of 1,3-β-glucan synthase (248). The mechanism by which *HKR1* may regulate 1,3-β-glucan synthesis is also unknown.

(ii) Genes defined by osmotic fragility. On the basis of the assumption that 1,3-β-glucan is essential for yeast cell integrity, a search for *S. cerevisiae* (408) and *S. pombe* (359) mutants that require the presence of an osmotic stabilizer to survive at nonpermissive temperatures has been reported. Early work allowed the isolation of a yeast mutant that lysed in hypotonic solutions (32, 437), but genetic and phenotypic characterization of the *srb1* locus has not been described. In addition, the isolation of thermosensitive lytic mutants (62) had afforded a mutant whose lysis was remediable by osmotic stabilizers, leading to the isolation of the *SLT2* gene (422). Slt2p belongs to a phosphorylation cascade led by a protein kinase C homolog that is thought to be essential for cell integrity (252, 289) (see below), but no direct involvement of this cascade in glucan synthesis has been reported so far. As well as the protein kinase mutants belonging to that cascade, there are a growing number of mutants that require osmotically stabilized medium. The genes have been identified as encoding key pieces in intracellular signal transduction pathways, including protein phosphatases and small G proteins from the Ras oncogene superfamily. These results offer a new way to explore the mechanisms that control cell wall synthesis and assembly and the interconnections of these mechanisms with other morphogenetic events (see the section on morphogenetic pathways, below).

Genes involved in 1,6-β-glucan synthesis. The yeast killer toxin is a small protein that binds to a 1,6-β-glucan-containing receptor in the yeast cell wall before making its way to the plasma membrane and producing a lethal effect (205). This binding is necessary for the toxin to kill sensitive yeast cells and permits a selection procedure for mutants resistant to the toxin that fail to make a functional receptor. In this way, a series of killer-resistant (*kre*) mutants partially defective in synthesis and assembly of that cell wall β-glucan have been isolated (see a list of the cloned genes in Table 2).

KRE5 is presumed to function early on the 1,6-β-glucan pathway (295). Disruption of *KRE5* induces a very low growth rate, which is not alleviated by the presence of an osmotic stabilizer, and cells appear with aberrant morphology and even lack of viability in some genetic backgrounds. Kre5p contains the N-terminal signal sequence for secretion and an HDEL C-terminal retention signal for soluble proteins residing in the endoplasmic reticulum in yeast cells (295).

The *KRE6* gene encodes a predicted type II membrane glycoprotein. *kre6* disruptants display a 50% reduction in the level of 1,6-β-glucan compared with the wild type (367). The *SKN1* gene was isolated as a multicopy suppressor of a *kre6* mutant (368), and it also encodes a type II transmembrane protein, which is highly homologous to Kre6p. *SKN1* disruption has no effect on killer sensitivity or 1,6-β-glucan levels. *skn1 kre6* double disruptants, however, show a drastic reduction in 1,6-β-glucan levels. It has been proposed that *KRE6* and *SKN1* would be redundant genes functioning in parallel in 1,6-β-glucan synthesis (368). *KRE6* or *SKN1* disruptions also lead to reductions in 1,3-β-glucan synthase levels as measured in vitro, but no further reduction is found in the double disruptant (368). The significance of this result is not known. Recent findings indicate that Kre6p is probably localized in a Golgi subcompartment and that both Kre6p and Skn1p are phosphorylated and therefore possibly regulated by phosphorylation/dephosphorylation (369). On the basis of genetic interactions, Roemer et al. propose that the protein kinase cascade led by Pkc1 could be a candidate for the regulation of their function (369) (see below and Fig. 6).

The *KRE11* gene has also been cloned (45). Its disruption is not lethal but leads to a 50% reduced level of 1,6-β-glucan, which is smaller than the one found in the wild type, although it contains a similar proportion of 1,3-β and 1,6-β linkages. Since *kre11 kre6* double disruptants are lethal, it has been suggested that Kre11p may be involved in the control of Kre6p function (45).

KRE1 encodes a serine/threonine-rich protein that appears to be heavily O glycosylated (36). It has an N-terminal signal sequence for secretion and a C-terminal hydrophobic domain containing a glycophospholipid anchor signal. The outermost proteinaceous electron-dense layer of the cell wall is missing in *KRE1* disruptant cells (36). This observation reinforces the hypothesis that mannoproteins are linked to the 1,6-β-glucan fraction of the wall (see the section on mannans, below).

Disruption of the *KRE9* gene, which encodes an O-glycosylated protein, produces an 80% reduction in the amount of 1,6-β-glucan. In addition, *kre9* null mutant cells are multibudded and have lost the laminar cell wall structure normally seen in wild-type cells. They also fail to form mating projections (44). *SKN7* is a multicopy suppressor of a *kre9* disrupted strain (46). It shows homology to bacterial two-component response regulators, and it is interesting that it is partially able to suppress the phenotype of protein kinase C mutants (see below) (47). Double mutations of *kre9* plus null mutations in the *KRE1*, *KRE6* or *KRE11* gene are lethal in an appropriate genetic background. Kre1p is located at the cell surface, and Kre9p, when overproduced, is detected extracellularly in the culture medium (37, 44).

KRE2/MNT1 encodes a 1,2-α-mannosyltransferase involved in O glycosylation (184, 185, 194). O-glycosylated proteins in budding-yeast cells have been shown to bear a linear polysaccharide chain of five residues (190, 420). Kre2p/Mnt2p is responsible for the addition of the third residue to that chain. Kre2p belongs to a family of Golgi transmembrane proteins, including Yur1p, Ktr1p, and Ktr2p, which may be involved in glycosylation of cell wall mannoproteins (274a). *PMT1* and

PMT2 are homologous genes that encode another mannosyltransferase activity that attaches mannose to peptidyl serine/threonine entering the secretory pathway (274, 411). Mutants with mutations in the *KRE2/MNT1* or *PMT1* genes are not apparently defective in the alkali-insoluble 1,6-β-glucan fraction but are killer resistant. The defect in 1,6-β-glucan is possibly located in other cell wall fraction in which β-glucan synthase and mannosyl transferase are both required. This type of chain may be involved in the cross-linking between β-glucans and cell wall mannoproteins (302, 433, 463) (see the section on mannans, below).

Precise functions, either catalytic or regulatory, have not been definitively assigned to any *KRE* genes (except for *KRE2* and *PMT1*). Nevertheless, on the basis of all the current information on these *KRE* genes provided by the team led by H. Bussey, it is reasonable to conclude that 1,6-β-glucan synthesis appears to operate along a secretory pathway. Integration of the *KRE* genes in an ordered pathway has been proposed (368). Recently, some new *kre* mutants have been detected among the Calcofluor white-hypersensitive mutants, *cwh*, mentioned above (353). Study of these novel mutants might add critical information in the future to our current knowledge of the biochemical events that govern 1,6-β-glucan synthesis. For a recent summary of *KRE* functions, the reader is referred to reference 231.

Mannan

Mannan is a yeast mannoprotein with a high mannose content that exists as covalent complexes with proteins. It constitutes one of the major cell wall components. According to their function, mannoproteins may be arbitrarily classified into at least two groups: (i) structural mannoproteins that form integral components of the cell wall, and (ii) enzymes located in the cell wall or in the periplasmic space (10), performing some trophic or morphogenetic role or simply in the process of being extracellularly secreted, such as invertase or acid phosphatase.

Cell wall structural mannoproteins are large molecules, containing about 95% carbohydrate, that can be extracted from the cell wall of intact cells without disturbing cell shape (294). In view of this, mannoproteins have been considered a filling material, enmeshed in the glucan structural network. However, the protein component plays a fundamental role in the process of biosynthesis as an acceptor of different sugar chains and a guide through the different organelles along the secretory pathway (323).

The structure of yeast mannan has been well established, mainly in the work of Ballou and coworkers (18). Understanding of the biosynthetic and secretional processes of mannoproteins has been accomplished mainly in studies on three classes of mutants: *mnn* (17, 18), *alg* (202), and *sec* (387). The attachment of sugar chains to the protein is mediated by either N glycosylation or O glycosylation (245, 420), although recently another glycosylation pattern occurring through the attachment of a glycosylphosphoinositol (GPI) anchor at the C-terminal end of membrane proteins has been described (92, 139). For an updated review about glycoprotein biosynthesis in yeast cells, the reader is referred to reference 190.

Cell wall mannoproteins. Cell wall mannoproteins can be extracted by reducing agents, detergents, and/or enzymatic digestion with different hydrolases (e.g., β-glucanases) (154, 340, 396, 428). Pretreatment of cells with a specific protease removes the outer cell wall mannoprotein layer and facilitates the subsequent lytic effect of the β-glucanase (463). Another alternative consists of using regenerating protoplasts (429), which excrete the mannoproteins into the medium, a method that should render additional information about the composition of cell wall precursors and their interactions.

It is difficult to separate mannoproteins, because most of them run as diffuse bands in electrophoresis gels because of the presence of hypermannosylated N chains. The use of specific tools such as tunicamycin—an N-glycosylation inhibitor—or specific endoglycosidases may be very helpful (335). The use of the *mnn9* mutant, in which N chains are considerably shortened, has allowed the identification of some definite bands after extraction of cell walls with 1,3-β-glucanase (433). Some mannoproteins are not sensitive to endo-β-*N*-acetylglucosaminidase H, an indication that they are only O glycosylated (334, 425).

Several cell wall proteins (structural mannoproteins) have been identified by studying their functions and cloning the corresponding genes, e.g., sexual agglutinins (266), flocculin (441), and mannoproteins involved in mating (Fus1p) (424, 425) or *S. cerevisiae* killer toxin resistance (Kre1p) (38). In other cases, the precise function is not yet known, i.e., Gas1p/Ggp1p (325, 426). Some other known mannoproteins (Exg Ia, Exg Ib, Exg II, gp29, and Cts1p) are cell wall hydrolytic enzymes located in the periplasmic space, and their gene structure and function are described in depth below.

Attachment of mannoproteins to the cell wall. Cell wall mannoproteins follow the secretory pathway and finally remain somehow trapped in the periplasmic space or in the glucan matrix. Even if mannoproteins were joined by disulfide bridges and/or hydrophobic bonds, this would not be sufficient to ensure their strong linkage to the wall matrix, as judged by the chemical procedures usually used for their extraction. Therefore, at least some of them must be covalently bound to other cell wall components, for instance to β-glucan.

Some mannoproteins extracted from wild-type yeast cells (396) or the *mnn9* mutant (433) by β-glucanase treatment contain a small amount of glucose that is tenaciously attached. The glucose residues cannot be eliminated by either endo-β-*N*-acetylglucosaminidase H or peptidyl *N*-glycosidase F digestion. Complete degradation of protein by alkaline hydrolysis releases not only the N- and O-glycosidically linked chains but also another one containing residual *N*-acetylglucosamine, mannose, and β-linked glucose (433). By using antibodies against 1,6-β-glucan, it is possible to detect some mannoproteins by Western immunoblot analysis (302). The structure of the 1,6-β-glucose-containing chains is unknown, but preliminary results indicate that they consist of about equal amounts of 1,6-β-glucose and 1,6-α-mannose residues. It has been suggested that Kre5p or Kre6p might be involved in the addition of the 1,6-β-linked glucose units (302), but the enzyme responsible for the nature of the interlinkage between the α-mannosyl and β-glucosyl residues is unknown, as is the nature of the linkage of these chains to the protein moiety. The extraction procedure of these mannoproteins implies degradation of 1,3-β-glucose linkages, and it is therefore reasonable to speculate that 1,6-β-glucose-containing side chains might be extended with 1,3-β-glucose residues. Thus, a covalently linked glucan-mannoprotein complex would exist in vivo.

Aculeacin A, an inhibitor of β-glucan synthesis, causes a decrease in the incorporation of mannoproteins to the wall and the excretion of a particular mannoprotein to the culture medium (429). On the other hand, a reduction in the cell wall galactomannan content has been described for a *S. pombe* spherical mutant resistant to papulacandin B (another inhibitor of β-glucan synthesis) (360); cosegregation of resistance and round morphology phenotypes is accompanied by oversecretion of galactomannoproteins into the culture medium

(150). These observations suggest failures in a presumptive mechanism for anchoring mannoproteins to the wall β-glucan.

A GPI anchor constitutes another mechanism by which some yeast cell wall mannoproteins remain attached to the cell surface. α-Agglutinin (273) and Gas1p/Ggp1p (325, 426) are examples in which the GPI anchor mechanism has been studied in detail. The GPI anchor glycan core consists of a residue of ethanolamine-phosphate-(mannose)$_3$-glucosamine-inositol attached to a membrane phospholipid (92). Transfer of the glycolipid to the protein takes place in the endoplasmic reticulum (93). The anchor is attached to the protein at the nonreducing end of the glycan chain and can be removed by a specific phospholipase C (148). GPI-anchored proteins have both a hydrophobic N-terminal signal sequence and a hydrophobic C-terminal peptide that is cleaved and replaced with the GPI anchor by a C-terminal transaminidase. The *GAA1* gene product is involved in this function (178). The determinants for GPI anchoring and the site of cleavage have been described previously (164, 324, 325). In short, these GPI-anchored proteins are synthesized as precursors with a serine-rich region, immediately followed by a pair of small residues (Ser, Gly, Ala, Asp, Asn, or Cys) positioned 10 to 12 residues N-terminal to a C-terminal domain composed mainly of hydrophobic amino acids. Anchor addition involves a coordinated processing event in which the precursor is cleaved at the level of the small residue motif, thus removing the hydrophobic C-terminal domain to the nascent protein, followed by the attachment of the GPI group to the new C terminus (69, 266). Elimination or alteration of the C-terminal domain in Gas1p/Ggp1p protein results in secretion of the protein to the medium (325). The isolation of temperature-sensitive mutants defective in GPI anchoring might help in the study of the fate of many cell surface mannoproteins (262). The *CWH6/SPT14* gene product, which was isolated by complementation of one of the Calcofluor white hypersensitive mutants (353), is probably responsible for the transfer of *N*-acetylglucosamine to a precursor of the GPI anchor (439a). A *cwh6* mutant shows a decrease of 50% in the amount of mannan in the cell wall (439a), suggesting that GPI anchoring is a key mechanism for the incorporation of an important number of mannoproteins into the yeast cell wall.

It is known that mature α-agglutinin is anchored to the cell wall rather than to the plasma membrane. Accordingly, it has been speculated that GPI anchorage of some proteins to the membrane might be an intermediate form in a process in which cross-linking with other cell wall components is finally achieved (112). In the case of α-agglutinin, this process has been demonstrated (273). Glycosylation and attachment to the *S. cerevisiae* cell wall of chimeric proteins extended with the C-terminal 30 amino acids of α-agglutinin have also been reported (431). Furthermore, anchoring of the chimeric proteins is apparently mediated by 1,6-β-glucan chains, as in the case of the mannoproteins mentioned above. Other GPI-anchored mannoproteins are probably cross-linked to the wall by the same procedure.

The mechanism of these cross-linking reactions is not known, but it may act in the periplasmic space by transglycosylation. Recently, several possibilities have been proposed (112). The existence of that type of reactions affords a new perspective about the role of mannoproteins in morphogenesis being more active than previously thought (392). In addition, these reactions may be considered selective targets for the development of new antifungal agents.

GPI addition to cell wall mannoproteins is necessary for subsequent cross-linking to other cell wall components but is not sufficient to specify cell wall location. Indeed, there is at least one mannoprotein, Gas1p/Ggp1p, that is apparently not associated with the cell wall but, rather, with the plasma membrane. Recent results suggest that *GAS1/GGP1* would be a gene with an important morphogenetic role. Disruption of *GAS1/GGP1* reveals several defects in the vegetative cycle (347). The cells are round, have reduced viability in the stationary phase, and display an increase and delocalization of chitin synthesis. More recently, it has been shown that *CWH52*, a gene that complements Calcofluor white hypersensitivity of the corresponding mutant, is identical to *GAS1/GGP1* (352). Transcription of *CWH52* is cell cycle regulated, and *cwh52* mutants have a reduced cell wall glucose content. Finally, strains with null mutations of *PHR1*, a pH-regulated gene of *C. albicans* that codes for a protein 56% identical to Gas1p/Ggp1p, exhibit a pH-conditional morphological defect (383). Under alkaline pH conditions, the mutant is unable to achieve apical growth and cells became rounded, highly enlarged, and multibudded, although actin distribution and secretion are not affected. Phr1p function either is not required at acidic pH or is performed by a homolog functioning at low pH (383). Phr1p and, by extrapolation, Gas1/Ggp1/Cwh52p may play an essential and specific role in directing apical growth. However, its extracellular location is consistent with a morphogenetic role related to cell wall assembly. It would be interesting to be able to assign an enzymatic function to Gas1p/Ggp1p.

CELL WALL HYDROLYTIC ENZYMES

Assembly of externalized cell wall components into a supramolecular organelle with specific morphology presumably occurs through extensive interaction among various enzymatic, chemical, and physical forces, whose nature is completely unknown. Two mechanisms to explain the incorporation of new wall material into a preexisting fabric have been proposed. One states that cell wall expansion may involve a continuous process of synthesis and degradation and that new building blocks are inserted at growth sites in which bonds in recently formed cell wall are cleaved. This notion of wall growth as a dynamic balance of lytic- and ligase-type activities (215) receives continued support, although definitive proof of this concept is still lacking. The second explanation is that new wall would grow by a process of intussusception before cross-links are made and rigidity sets in; cell wall polymers, which at the growing points may not be completely solidified by cross-links, slide apart, thus providing an area for insertion of new material. There are, however, instances in which it is necessary that old (presumably finished) wall be broken, such as bud emergence. In this case, it seems imperative to soften the old wall by some hydrolytic process to allow emergence of the new growth (61, 64).

Whatever the mechanism, it seems clear that at some moments, a highly localized breakage of the existing wall occurs, and, consistent with this, a number of autolytic hydrolases have been reported to be associated with the yeast envelope, including glucanase, chitinase, and α-mannosidase activities.

The genes associated with cell wall hydrolysis are listed in Table 3.

Glucanases

As mentioned in previous sections, 1,3-β-glucan constitutes the main structural polysaccharide responsible for the shape and rigidity of the yeast cell envelope. Although the yeast cell wall glucans do not undergo appreciable turnover during vegetative growth (240, 439), limited site-directed hydrolysis of the rigid skeletal wall β-glucans, mediated by endogenous β-glucanases, probably takes place during various morphogenetic

TABLE 3. Genes involved in cell wall hydrolytic pathways

Gene (synonym)	Chromosomal location	Enzyme activity	Regulation	Cellular location	References
EXG1 (*BGL1*)	12R	Exoglucanases Exg Ia and Exg Ib[a]	Constitutive, osmosensing?	Periplasmic space and medium	214, 436
EXG2	4R	Exoglucanase Exg II[b]	Constitutive	Cell wall and medium	94, 314
BGL2	?	Endoglucanase (gp29)	Constitutive	Cell wall	230, 304
SSG1 (*SPR1*)	15R	Sporulation exoglucanase	Meiotic program	Ascospore wall	88, 308, 379
CTS1	?	Endochitinase	Constitutive (cell cycle)	Cell wall and medium	118, 247
AMS1	7L	α-Mannosidase	Catabolite repression	Vacuolar membrane	246

[a] Formerly Exg I and Exg II, respectively.
[b] Formerly Exg III.

processes, such as budding, wall growth, conjugation, and ascus formation (316). Two broad classes of 1,3-β-glucanase activity occur in yeasts, namely, the exoglucanases and the endoglucanases. As measured by their activity on the substrate laminarin (a linear 1,3-β-glucan), exo-1,3-β-glucanases account for the greater part of total glucanase activity in yeasts and hydrolyze the β-O-glycosidic linkages at the nonreducing end of the polymer chain, resulting in the release of glucose. These enzymes are not particularly specific, because they usually act also on 1,6-β-linkages (as measured against pustulan, a linear 1,6-β-glucan substrate), although with less efficiency. Endo-1,3-β-glucanases attack the linkages at intermediate points of the polymer chain, releasing a mixture of oligosaccharides with glucose as a minor product.

For a number of years, the complement of glucan-hydrolyzing enzymes in vegetatively growing *S. cerevisiae* cells seemed to be well characterized and simple and was attributed to three glycoprotein forms, synthesized constitutively, which are first secreted to the periplasmic space and then released into the culture medium, where they accumulate. The major form is an exo-1,3-β-glucanase (formerly Exg II and hereafter Exg Ib) that hydrolyzes both 1,3-β- and 1,6-β-linkages (the latter to a lesser extent) and even has β-glucosidase activity, since it also acts on synthetic glucosides such as *p*-nitrophenyl-β-glucoside and 4-methylumbelliferyl-β-glucoside. The second form is also a nonspecific exo-β-glucanase (formerly Exg I and hereafter Exg Ia), which has an identical protein portion to the major exo-β-glucanase, although it differs in having a higher content of the carbohydrate moiety. Finally, a high-M_r endo-1,3-β-glucanase is also present but in smaller amounts (30, 138, 377). In turn, the meiotic cycle leads to the induction of a new exo-1,3-β-glucanase which is not present in vegetatively growing cells and which is therefore specifically associated with the sporulation process (109, 110).

Current knowledge, however, suggests that the yeast glucanase system is more complex and points to the existence of minor forms, some being present in very small amounts, which might be secreted to remain in the cell wall rather than in the periplasmic space or to be released into the culture fluid. Thus, using mutant strains defective in the aforementioned Exg Ia and Exg Ib exo-β-glucanases, Cenamor et al. (72) reported two additional glucan-hydrolyzing enzymes. These glucanases, detected in protoplast lysates but undetectable in cell extracts and in culture fluids, are secreted by protoplasts under regeneration conditions, when many of the secreted proteins cannot become entrapped in the wall structure. Along the same lines, Klebl and Tanner (230) purified a tightly bound cell wall glycoprotein, with an apparent molecular mass of 29 kDa (gp29), which specifically binds to insoluble *S. cerevisiae* glucan and chitin and was shown to exhibit endo-1,3-β-glucanase activity. Interestingly, this cell wall-associated enzyme is widely distributed and highly conserved among yeasts, since proteins related to gp29, with M_rs ranging from 31,500 to 34,000 depending on the species, have been detected by immunoblotting in *C. albicans*, *Hansenula wingei*, *Torulopsis glabrata*, *Pichia guilliermondii*, and *K. lactis* (180, 188, 230).

Genetic characterization. In recent years, much work has focused on the characterization of the genetic system governing 1,3-β-glucanase synthesis in yeasts. Genetic approaches were initiated by the isolation of *S. cerevisiae* mutants with highly reduced exo-β-glucanase activity by a screening method based on the capacity of these enzymes to hydrolyze synthetic derivatives, such as the fluorogenic substrate 4-methylumbelliferyl-β-glucoside (381). By functional complementation of the chromosomal mutation harbored by these strains (*exg1*), it was possible to clone and identify two different fragments that were able to restore the capacity to hydrolyze MUG. One of the fragments contains a structural gene (*EXG1*) coding for a protein whose differential glycosylation accounts for the two main extracellular exo-1,3-β-glucanases (Exg Ia and Exg Ib) in culture supernatants of *S. cerevisiae*. The second fragment identified produces only partial complementation of the Exg$^-$ phenotype of *exg1* strains and contains a gene (*EXG2*) that codes for a minor exo-1,3-β-glucanase exhibiting a high carbohydrate content, which has been designated Exg II (formerly Exg III) (313, 314, 354).

EXG1, which has also been cloned selecting for overproducers (*BGL1*) (246), is 1,344 nucleotides in length and encodes a 51.3-kDa polypeptide which contains two potential N-glycosylation sites. The putative precursor contains an N-terminal extended peptide of 40 amino acids that is cleaved off during the process of protein externalization. Since the cleaved site is preceded by a pair of basic amino acid residues (Lys-Arg), which is one of the proteolytic processing signals of a trypsin-like endoprotease encoded by the *KEX2* gene, this finding raises the possibility that these exo-β-glucanases (Exg Ia and Exg Ib) are synthesized as a precursor protein whose maturation involves the removal of the signal peptide by the signal peptidase during translocation to the endoplasmic reticulum, followed by proteolytic processing in the Golgi complex by the *KEX2* endoprotease. It is interesting that although this processing has been shown to occur in extracellular polypeptides, such as α-factor or the killer toxin, it has not been described for any of the other secreted glycoproteins with enzymatic activity, such as invertase, α-galactosidase, or the acid phosphatases, which are also targeted to the cell surface and follow the same secretory pathway (22, 436).

Sequencing analysis of the *EXG2* gene shows a single open reading frame of 1,686 bp encoding a 63.5-kDa polypeptide which contains 14 tripeptide sequences, Asn-X-Thr or Asn-X-Ser, whose N-glycosylation may account for the high molecular mass of the mature enzyme (~200 kDa). Interestingly, com-

parison of the primary translation products encoded by *EXG1* and *EXG2* reveals that both proteins are extremely homologous (41% similarity with conservative substitutions) (94, 95, 111). As in the case of the *EXG1* product, the N-terminal region of the putative precursor encoded by the *EXG2* gene is a very hydrophobic segment with structural features resembling those of signal peptides of secreted proteins. In contrast to the *EXG1*-encoded glucanase, which is efficiently secreted to the periplasmic space and then released into the surrounding medium, only about 5% of the *EXG2*-encoded exo-1,3-β-glucanase (Exg II) is detected in the culture supernatant. Subcellular fractionation experiments show that most of the cell-retained activity is associated with the cell wall structure. Comparison of the predicted amino acid sequence of this exo-1,3-β-glucanase with those of other reported yeast cell wall-associated proteins reveals a C-terminal structure characteristic of polypeptides attached to the plasma membrane through a GPI anchor (see above) (268, 325, 374). As in the case of Gas1p/Ggp1p, a truncated Exg II mutant protein lacking the 69 C-terminal residues, including the serine-rich region, the putative GPI attachment site (KNAA), and the hydrophobic C terminus, is not localized in the cell wall structure but, rather, is mostly released into the medium, thus pointing to a direct involvement of the C terminus of the primary translation product in the correct location of the mature wild-type Exg II 1,3-β-glucanase (94).

The gene (*BGL2*) for the gp29 cell wall-associated endo-1,3-β-glucanase was molecularly cloned by screening a genomic library for hybridization with synthetic oligonucleotides deduced from the N-terminal sequence of the native protein. *BGL2* encodes a polypeptide of 313 residues with a typical 23-amino-acid signal peptide and two potential N-glycosylation sites, only one of them bearing oligosaccharide chains attached to the asparagine residue (230, 304). As mentioned above, the gp29 glucanase exhibits an endo-hydrolytic mode of action, this peculiarity being reflected in the primary structure of the protein when compared with the *EXG1*- and *EXG2*-encoded exoglucanases. Alignment of the predicted sequence of the mature proteins encoded by *BGL2* and *EXG1* does not reveal a significant degree of homology; instead, the *BGL2* product displays considerable similarity to plant 1,3-β-glucanases, such as those from barley aleurone and tobacco cells (140, 398).

Two groups have independently isolated and characterized the gene (*SSG1*/*SPR1*) encoding the sporulation-specific exo-1,3-β-glucanase. *SSG1* was cloned by screening a genomic library with a DNA probe obtained by PCR amplification with synthetic oligonucleotides designed according to the nucleotide sequence predicted from the N-terminal region of the purified protein (379). In an alternative approach, *SPR1* was identified by differential hybridization screening for genes transcribed preferentially during sporulation (88, 308). *SSG1* is 1,335 nucleotides in length and encodes a 51.7-kDa polypeptide, which contains one potential N-glycosylation site and exhibits a strong homology to the *EXG1*-encoded polypeptide (76% similarity with conservative substitutions). Northern (RNA) blot analysis discloses a unique *SSG1*-specific transcript which is present only in sporulating diploids (*MAT***a**/*MAT*α) and does not appear in vegetatively growing cells or in nonsporulating diploids (*MAT*α/*MAT*α) when incubated under nitrogen starvation conditions. The meiotic time course of *SSG1* induction, monitored by Northern analyses carried out with *MAT***a**/*MAT*α cells at various times during the sporulation process, indicates a temporal pattern of expression that resembles that of the previously described late class of sporulation-specific transcripts, appearing at the time of meiosis I and reaching a maximum during spore formation (195, 219).

Assessing a biological function for yeast glucanases. The large body of information currently available concerning at least some of the genes involved in this complex system has now opened the way for molecular biology approaches based on the isolation and characterization of mutants altered or defective in β-glucanase production. The expectations generated, however, have not been reflected in direct experimental evidence for the biological role of these proteins in controlled modifications of the yeast cell envelope. Characterization of chemically induced *exg1* mutants led to the unexpected demonstration that the Exg Ia and Exg Ib enzymes, by far the most abundant β-glucanases in *S. cerevisiae*, do not play an essential role in morphogenesis, since cells devoid of this activity were able to grow, mate, and sporulate normally (381, 382). It was argued that tiny, undetectable amounts of exo-1,3-β-glucanase activity, sufficient to accomplish its biological role, could still be produced because of leakiness of the mutation. Deletion of the *EXG1* gene, however, confirmed the dispensability of these enzymes, because *exg1* null mutants behave in the same way as the parental strains with respect to all features investigated, such as growth, osmotic stability, mating, and sporulation of the homozygous diploid (314). As an explanation, it was argued that the *EXG1*-encoded glucanases are simply β-glucosidases that happen to act on 1,3-β and 1,6-β linkages of glucan but are produced for metabolic and not for morphogenetic purposes (361). However, the certitude of this conjecture is questioned by two facts: (i) Exg Ia and Exg Ib production is growth associated (108, 331), unlike that of metabolic hydrolases, which are usually subjected to catabolite repression; and (ii) overexpression of *EXG1* causes a killer toxin resistance phenotype reminiscent of that of the *kre* mutants (214), suggesting that 1,6-β-glucan may be an in vivo target for Exg Ia or Exg Ib activities.

The lack of a drastic defect on *EXG1*-disrupted strains may simply be a reflection of the multiplicity of glucan-hydrolyzing enzymes and could mean that the cell might dispense some of them with no significant alterations. Although functionally redundant proteins do not necessarily have to be encoded by homologous genes, the strong similarity of the *EXG2*-encoded exo-1,3-β-glucanase to those encoded by the *EXG1* gene would support the notion of the presence of gene products that could serve complementary functions such that the cell would be able to compensate for the absence of one of them. The behavior of doubly disrupted strains (*exg1 exg2*) clearly rules out such possibility, although one cannot totally rule out the possibility of a functional complementation since these mutants still retain exo-1,3-β-glucanase activity, probably because of a minor, as yet unidentified, form (94, 379).

Because of their mechanism of action on the substrate, it is also conceivable that endoglucanases are the enzymes relevant for the growth and development of cells and that exoglucanases are only accessory or even dispensable. The reduced body of evidence available for *S. cerevisiae* and other yeast species suggests that exoglucanases are not able to hydrolyze isolated yeast walls or wall glucans (144, 193). By contrast, some endoglucanases completely solubilize or lyse isolated walls (145, 357, 438), and this finding implies the presence of certain linkages accessible only to endoglucanase cleavage that are critical for the maintenance of wall integrity. The hydrolytic mode of action exhibited by the *BGL2*-encoded product, together with its lectin-like binding capacity to yeast 1,3-β-glucan, makes this glucanase a good candidate for playing such a crucial role in cell morphogenesis. *BGL2* disruption, however, does not result in a phenotype under laboratory conditions, and mating and sporulation are not affected; only gp29 overproducers, by transformation with a high-copy-number plas-

mid containing *BGL2*, grow considerably more slowly than control cells. Therefore, the function of the gp29 endo-1,3-β-glucanase in null mutants seems to be compensated, while larger amounts of the protein within the cell are at least inhibitory for growth (304).

Confinement of *SSG1* gene expression to sporulation, together with its timing of production in the ordered sequence of events which constitutes the meiotic process in *S. cerevisiae*, is suggestive of a direct participation of the encoded 1,3-β-glucanase in morphogenetic events related to ascospore wall development. Because sporulation in *S. cerevisiae* can occur on potassium acetate alone, it seems that extensive recycling of the parent cell wall components is required to provide precursors for the synthesis of spore macromolecules, and since the appearance of the sporulation-specific exo-1,3-β-glucanase occurs largely before the majority of ascospores have actually formed, it may well have a metabolic function directed toward the mobilization and organization of vegetative cell wall 1,3-β-glucan into ascospore walls. Thin-section electron microscopy of asci in *S. cerevisiae*, however, does not suggest that the parent wall itself is extensively reutilized (153), and recycling is probably restricted to cellular contents (199, 221). A more direct involvement of the *SSG1*-encoded product in the construction of the ascospore cell wall should then be considered. Despite this, whatever its physiological function, it is clear that *SSG1* is not essential for successful completion of sporulation, and homozygous *ssg1*/*ssg1* diploids undergo meiosis and give rise to four apparently normal, viable ascospores (308, 379). This is not surprising if one takes into account that previous mutational analyses of several other genes that display a late sporulation-specific expression pattern in most cases failed to reveal dramatic effects on spore formation. Thus, with the exception of the *SPS1* gene, whose disruption has been shown to give rise to an asporogenous phenotype (343), nonessential functions have been attributed to the products encoded by several late genes such as *SGA1* (451), *SPS2* (343), *SPS4* (160), *LGN2* (170), *SPR2*, *SPR6*, and *SPR9* (220, 224).

A search for a more subtle defect, however, shows differences between the mutant and wild-type strains, pointing to the possibility that the *SSG1*-encoded 1,3-β-glucanase serves some important but not indispensable function in the formation of ascospores. Thus, analysis of the time course of ascus formation reveals a delay in the appearance of asci in the *SSG1*-disrupted strain such that after 16 h in sporulation medium, about half of the wild-type diploid cells have been converted into asci whereas only one-quarter of the homozygous mutant population have sporulated; these differences are reduced by longer incubation periods, reaching almost similar percentages at 37 h, when ascus formation is nearly completed (379). Complementary results were obtained on testing the capacity of ascospores derived from *SPR1*-disrupted diploids to survive heat stress: mutant spores lose viability at 42°C much more rapidly than do similarly treated wild-type spores (308). This phenomenon seems most likely to be attributable to a delay in spore wall maturation, because the increased killing rate is observed in spores only from newly formed asci (up to 20 h in sporulation medium) and not in mature (24 h or older) asci. The observation that wild-type and mutant spores obtained by sporulation of a heterozygous (*SPR1*/*spr1*) diploid strain were killed at equal rates at 42°C (308) suggests that the sporulation exo-1,3-β-glucanase functions before partitioning of nuclei or is distributed to developing ascospores prior to the completion of prospore walls.

On the basis of the significant degree of similarity between the *SSG1*-encoded product and the 1,3-β-glucanases encoded by the *EXG1* and *EXG2* genes, the possibility that the lack of a phenotype severely defective in spore formation in *SSG1*-disrupted strains is the result of overlapping activities by vegetative 1,3-β-glucanases is really attractive. Functional redundancy has also been invoked to explain why mature spores from homozygous *SPS100*-disrupted diploids are as resistant to ether as are wild-type controls, despite there being a significant delay in the onset of ether resistance in developing mutant asci, and also to account for the failure to readily detect a mutant phenotype for other sporulation-specific genes such as *SPS2*, *SPS4*, and *SPS101* (160, 249, 342). The sporulation efficiencies of doubly and triply disrupted strains clearly show that this is not the case (379), although one cannot totally rule out the possibility of a functional complementation. Thus, the fact that the triple mutant still retains most of the glucan-degradative capacity exhibited by the singly *SSG1*-disrupted mutant points to both the small contribution of the *EXG1*- and *EXG2*-encoded products to the total 1,3-β-glucanase content during sporulation and, even more interestingly, the presence during this process of other 1,3-β-glucan-hydrolyzing enzymes. At present, it is not possible to elucidate which enzymatic forms might be responsible for these levels of 1,3-β-glucanase activity. On one hand, it should be taken into account that as mentioned above, apart from the *EXG1*- and *EXG2*-encoded 1,3-β-glucanases, other minor exo- and endo-1,3-β-glucanases have been described as occurring during vegetative growth (72) and their synthesis could be maintained or even activated during the meiotic program. Alternatively, some enzymatic form with exo- or endo-1,3-β-glucanase activity might exist, whose induction, like that of the *SSG1*-encoded product, is specifically coupled to sporulation events.

To conclude, it seems clear that unravelling of the biological function of each of the components of such a complex system demands a deeper study of their in vivo action through a systematic search for discernible phenotypes; this could lead, in some cases, to modifying former concepts created on the basis of in vitro analyses of alternative substrates with well-defined structures (such as laminarin or pustulan). As an example, the recent finding that overexpression of *EXG1* from a 2μm plasmid leads to a cell wall 1,6-β-glucan reduction and causes killer resistance in wild-type cells while *EXG1* deletion results in slight increases in killer sensitivity and 1,6-β-glucan levels (214) is a good indication that the most prevalent in vitro activity of these enzymes (1,3-β-glucanase) on a model substrate may not be a reflection of their mechanism of action on the native polymers. Two possible, not mutually exclusive, explanations have been invoked to explain these observations (214). Exo-β-glucanases hydrolyze glucan polymers from the nonreducing end, so that the *EXG1*-encoded Exg Ia and Exg Ib activities would preferentially break down the smaller and highly branched 1,6-β-glucan over the long linear 1,3-β-glucan. Alternatively, it could be speculated that these enzymes would function specifically as 1,6-β-glucanases on the in vivo cell wall glucans. Whatever the mechanism, demonstration that changes in Exg Ia and Exg Ib activity lead to detectable in vivo alterations in the cell wall provides the first experimental support for the expectation that *EXG1*-encoded glucanases play a functional role in cell wall glucan metabolism.

Chitinase

As described above, chitin is quantitatively a very minor wall component, although its specific deposition in the region of the septum is important for the mechanical stability of this temporary junction between mother and daughter cells. Degradation of this chitinous septum seems imperative to allow the daughter cell to begin an independent existence, but, in con-

trast to the situation with 1,3-β-glucanases, to date only one endochitinase which hydrolyzes both insoluble chitin and soluble substrate analogs has been found in *S. cerevisiae* (96, 132). Cloning of the gene for this enzyme, *CTS1*, was achieved by plasmid-based overexpression (246). The reported sequence predicts a 562-amino-acid protein with four clearly distinct domains: a signal peptide, a catalytic domain, a serine/threonine-rich region, and a carboxy-terminal domain with high binding affinity for chitin (247).

Although the majority of chitinase activity produced in yeast cultures (90%) is secreted into the growth medium, most of the cell-associated chitinase activity remains sequestered in the cell wall (60%). That portion of the enzyme that becomes associated with the cell wall apparently functions in cell separation, since *CTS1* disruption results in a clear inability of cells to separate normally. This block in cell separation is manifest in large aggregates of cells attached by their cell septum regions. Deletion of the C-terminal chitin-binding domain from a *CTS1* plasmid results in a decreased ability of the gene to complement the separation-defective phenotype of a *CTS1* disruption. These data suggest that the chitin-binding region functions in localizing the enzyme to the cell wall, where it hydrolyzes chitin fibers which join mother and daughter cells (247).

A clumpy phenotype similar to that observed in chitinase-deficient mutants is observed in strains harboring an *ace2* mutation, in which *CTS1* mRNA levels are drastically reduced. The *ACE2* gene is homologous to the *SWI5* gene, which encodes a zinc finger protein that functions as a cell cycle activator of the *HO* endonuclease gene transcription to initiate mating-type interconversion; the zinc finger DNA-binding domains of these two proteins are very highly conserved, with 85% identity in amino acid sequence (54, 409). Transcriptional studies on synchronous cells indicate that *CTS1* is expressed throughout the cell cycle with a periodicity of RNA accumulation similar to that of the *HO* gene (118). Maximal expression from these two genes occurs slightly before that of histone H2A, a marker of the interval between late G_1 phase and early S phase of the cell cycle.

ACE2 displays a pattern of cell cycle-regulated transcription (expression in the G_2 phase) and subcellular localization identical to that seen for *SWI5*, although, despite their similarities, *SWI5* and *ACE2* function in separate pathways of transcriptional regulation. *SWI5* is a transcriptional activator of the *HO* endonuclease gene, whereas *ACE2* is not. By contrast, *ACE2* is a transcriptional activator of the *CTS1* gene, whereas *SWI5* is not. Overproduction of either *SWI5* or *ACE2*, however, allows transcriptional activation of the target gene from the other pathway, suggesting that Ace2p and Swi5p both recognize the same DNA sequence. The fact that the *HO* gene is expressed in only one of the two cells produced from mitotic division and the many analogies between the *SWI5*/*HO* and *ACE2*/*CTS1* regulatory pathways, have led to the interesting suggestion that the *CTS1* gene, like *HO*, will be asymmetrically expressed in mothers and daughters, such that chitinase would be synthesized in only one of the progeny from cell division. The asymmetry of the bud scar is consistent with this notion (118).

Mannanases

Although mannan is a major wall component and it has been shown by pulse-chase experiments that at least a portion of the mannoprotein pool is subject to degradative turnover (339), there is only limited information on the system responsible for the breakdown of mannose-containing biopolymers from yeast cells. On the basis of knowledge of mannoprotein structure and studies with bacterial enzymes (16), one might expect exo-mannanase and endo-1,6-α-mannanase to be involved in the hydrolysis of mannan polysaccharide and an endo-β-*N*-acetylglucosaminidase to cleave the connection between polysaccharide and protein.

Several investigators have reported partial purification of a nonspecific α-mannosidase from *S. cerevisiae* that is active toward *p*-nitrophenyl-α-mannopyranoside (213, 226, 332). This activity has been reported to be a component of the vacuolar membrane and has been cataloged as a marker enzyme for this organelle (432). α-Mannosidase levels are apparently controlled by catabolite repression, since the enzyme concentration varies inversely with the growth rate, and have been shown to increase significantly in sporulating diploids until ascospores are completely formed (332).

The putative structural gene for this α-mannosidase (*AMS1*) was cloned by plasmid-based overexpression (246) and was found to encode a 1,083-amino-acid polypeptide with a calculated molecular mass of 107 kDa, containing seven potential N-glycosylation sites (458). No readily apparent phenotype is associated with α-mannosidase deficiency in vegetatively growing *AMS1*-disrupted strains, and since homozygous *AMS1* null mutants are able to sporulate normally, it appears that this hydrolase is not essential for the meiotic process. Labelling experiments with *AMS1* and *ams1* haploids and [2-^{3}H]mannose, however, suggest that the cloned α-mannosidase may play a role in glycoprotein metabolism, analogous to that of the corresponding hydrolase in mammalian lysosomes, by catalyzing the sequential removal of mannose residues from the nonreducing end of mannan chains (246).

MORPHOGENETIC PATHWAYS

Thus far, we have addressed what is known about the enzymatic machinery required for cell wall formation during the different stages of the budding-yeast life cycle. Now we shall consider the regulatory pathways that may control the timing and positioning of morphogenetic events at particular points in the cell cycle. As described above, certain cell wall biosynthetic enzymes are required only during a particular stage of the life cycle (Fig. 3). Very little is known about the transcriptional and/or posttranslational controls that lead to accumulation or activation of these enzymes at the proper stage or how such regulatory systems become activated by environmental changes, such as nitrogen starvation, the presence of mating pheromones, or intracellular signals from cell cycle control genes. Furthermore, there is an additional complication, namely, the requirement for an accurate polarization of cell wall components and enzymes to a discrete point under the cell surface. Secretion pathways, involving cytoskeletal proteins, have been shown to play a key role in polarized growth in yeasts. The signals for controlling such polarization are a matter of interest for many researchers, whose efforts have revealed an array of genes involved in these controls (74, 78, 277) and have provided important clues for our understanding of these processes, although the mechanism of polarization is still not well understood.

Connecting the Mitotic Cycle to Morphogenesis

Morphogenetic events during the *S. cerevisiae* cell cycle may be classified into four main levels: (i) bud site selection, which occurs in the G_1 phase; (ii) bud emergence, which is preceded by the localization of the actin cytoskeletal apparatus and other elements at the preselected point and occurs just after START; (iii) bud growth, which occurs from late G_1 through S and G_2 phases and involves a switch in actin localization from an apical

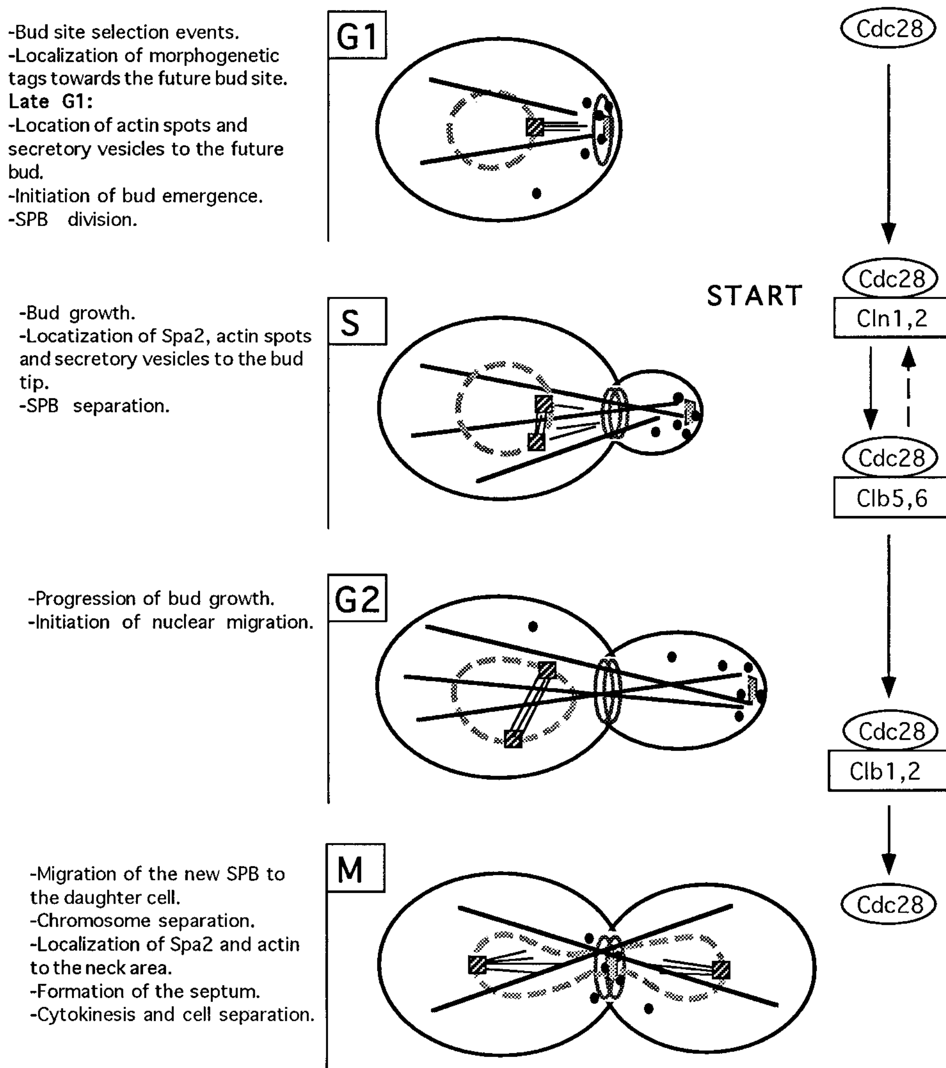


FIG. 4. Cell division cycle and morphogenesis. Coordination of nuclear and cytoskeletal dynamics with cell surface growth events, and their correlation with the binding of protein kinase Cdc28p to cyclins. Symbols: ▨, SPB; ----, nuclear membrane; ——, microtubules; ▬▬, actin cables; ⦶, 10-nm microfilament ring; ▯, Spa2 patch; ●, actin spot.

to a more diffuse pattern; and (iv) cytokinesis and cell separation, which involve a prior redistribution of the actin cytoskeleton to the septum area and occur after mitosis. Each of these stages is genetically programmed and synchronized with the nuclear events (Fig. 4). Synchrony between nuclear and cortical events is necessary for successful completion of the cell cycle, but many aspects of bud formation are relatively independent of those involving nuclear division. As an example, conditional mutations that block budding at early stages of cell cycle (*cdc24* or *cdc42* mutations [see below]) still allow nuclear division (183, 216, 404), although after a considerable delay in G_2 (260a).

Morphogenetic and spindle pole body (SPB) duplication processes are probably triggered by a common signal, since both begin simultaneously in late G_1, at the time of START in the cell cycle (350). The protein kinase activity of the *CDC28* gene product (the counterpart of Cdc2 in other eukaryotes), when bound to a family of regulatory proteins called cyclins, is known to be crucial for the commitment of cell cycle and entry into mitosis (355, 356, 449). When Cdc28p is bound to G_1 cyclins (encoded by the budding yeast *CLN* genes), morphogenetic events leading to bud emergence and SPB duplication occur. These events run parallel to the expression of proteins required for the entry of cells into the S phase (218). A different set of cyclins—*CLB3*, *CLB4*, *CLB5*, and *CLB6*—also present in early stages of the cycle may account for the modulation of the specific functions of *CDC28* in the commitment to the S phase (136, 244, 311, 390). The question now arises whether the cytoskeletal rearrangements leading to polarized secretion and bud initiation are also triggered by Cdc28-mediated phosphorylation.

During the G_1 phase, a bundle of microtubules emanating from the SPB (the yeast microtubule-organizing center) extends toward the future bud site (2, 212). In the submembrane at the incipient bud site, we find some intriguing elements: a patch of a nonessential protein that localizes at sites of cell growth (Spa2p) (405), and a ring of 10-nm microfilaments (thought to consist of *CDC3*, *CDC10*, *CDC11*, and *CDC12* gene products) which remains at the daughter-mother junction until cytokinesis and is probably responsible for some aspect of

TABLE 4. Genes related to the control of polarized growth and cell polarity

Gene	Encoded protein	Mutant phenotype	Reference(s)
CDC42	Ras-like GTPase	Unbudded multinucleated cells; delocalization of actin and chitin	1, 24, 183, 216, 460, 462
RHO1	Ras-like GTPase	Growth defect	276, 351, 452
RHO2	Ras-like GTPase	No defects	276, 351
RHO3	Ras-like GTPase	Growth defect	290, 291
RHO4	Ras-like GTPase	No defects	290, 291
CDC24	Ras-like GEF (for Cdc42)	Like *cdc42*	24, 183, 297, 298, 404, 459
BEM1	SH3 domain-containing protein	Budding and mating defect	25, 79, 80
BEM2	Rho GAP (for Rho1?)	Loss of polarity; budding defect (large multinucleate cells)	25, 229, 345, 461
BEM3	Ras-like GAP (for Cdc42)	No defects	460, 461
CDC43	Type I GGTase β-subunit	Like *cdc42*	1, 183, 292, 328, 330
RSR1 (*BUD1*)	Ras-like GTPase	Loss of polarity	24, 75, 76, 459
BUD2	GAP for Rsr1	Loss of polarity	28, 75, 76, 101, 338
BUD5	GEF for Rsr1	Loss of polarity	74–76
BUD3	?	Loss of axial budding	75, 76
BUD4	?	Loss of axial budding	75, 76
AXL1	Protease	Loss of axial budding	156
RAX1	Axl1 target?	Loss of axial budding	156
SPA2	?	Loss of polarity; mating defect	80, 162, 405, 406
CDC3, *CDC10*, *CDC11*, *CDC12*	Septins (putative constituents of the 10-nm microfilament ring)	Loss of polarity, hyperpolarized growth, and septation defects	87, 146, 152, 173, 182, 228
MSB1	?	No phenotype	24, 25
MSB2	Transmembrane protein	No phenotype	24, 26
CMD1	Calmodulin	Like *cdc42* (some alleles)	43, 327, 413[a]
AFR1	?	Defect in mating projection formation	235, 236

[a] Only work relative to the role of calmodulin in polarized growth control is cited.

this event (56, 152, 173, 228) (Fig. 4). Both elements could constitute part of a label system for bud site selection (146) which is established from the previous cell cycle (see below). Inside the area encircled by this ring, and as well as Spa2p, is located a series of proteins, as detected by immunofluorescence techniques. These include small GTPases of the Ras superfamily (Cdc42p, Rho1p, and Sec4) (321, 452, 462), unconventional myosin (Myo2p) (264), calmodulin (Cmd1p) (43, 413), and the SH3 domain-containing protein Bem1p (cited in references 78 and 260) (genes related to bud site selection and bud emergence are listed in Table 4). Also in late G_1, the "actin-cortical" or "actin-dots" cytoskeleton, consisting of F-actin and some actin-binding proteins (see below) (2, 227, 319), is localized at the future bud site. Long cytoplasmic actin cables also appear to point to the incipient bud site (2, 227), where secretory vesicles start to accumulate (55).

It is likely that START triggers some aspects of this polarization process. Using cells synchronized by centrifugal elutriation, Lew and Reed (259) found that prominent actin polarization occurs after START, indicating that the phosphorylating activity of Cdc28/G_1 cyclin is important for actin distribution at the future bud site, although it is likely that the site had already been chosen by the cell earlier in G_1 (278). Our current view is that the mechanisms for bud site selection depend on signals laid at the poles by remnants from the previous cell cycle acting as tags (see the section below) (74, 278); the START signal then triggers actin polymerization and secretion toward the preselected point in late G_1 (see Fig. 8).

A strong link between G_1 cyclins and bud emergence was recently identified when the Cross and Nasmyth teams simultaneously found that a bud site selection gene, *BUD2* (see below), is necessary for budding only in *cln1 cln2* mutant backgrounds (28, 101). This suggests that Cln1p and Cln2p would be involved in the control of morphogenetic events.

In the earlier stages of bud emergence, actin, Spa2p, Rho proteins, calmodulin, and secretory vesicles localize at the tip of the growing bud (2, 43, 405, 413, 452, 462). However, as the bud enlarges, the actin spots reduce their accumulation at the bud tip, scattering more diffusely around the bud (2, 278) and most of the aforementioned products are no longer conspicuous at the bud polar end, with the exception of Spa2p and Rho1p, which remain longer (43, 405, 452, 462). Growth remains active until the bud reaches its final size before mitosis. At this point, all these different components are either not visible or dispersed throughout the bud. After mitosis, cell wall synthesis is required again, this time to culminate the cycle in cell separation. At the time of cytokinesis, Spa2p, calmodulin, and actin redistribute to the septum zone, inside the area encircled by the outer chitin ring (43, 227, 405, 413).

Depolarization of the actin cortical cytoskeleton at the bud tip coincides with the activation of the Cdc28 kinase by a different family of cyclins, which are encoded by the *CLB1* and *CLB2* genes (165, 414). When activated by the Clb proteins, the Cdc28 kinase constitutes the yeast equivalent of maturation-promoting factor, which is found in higher eukaryotes and is responsible for the commitment of the nuclear changes that lead to chromosome segregation. Lew and Reed (259) also demonstrated that activation of the Cdc28/Cln1,2 complex is linked to the depolarization of actin at the bud tip, reporting that a lack of its activity resulted in hyperpolarized secretion leading to elongated buds. They also stated that overproduction of these cyclins accelerates the depolarization of actin in the daughter cell. These authors call this cycle-regulated depolarization the apical/isotropic switch (260), because it promotes a shift from the initial apical growth to an isodiametric enlargement of the bud.

Finally, inactivation of Cdc28p after mitosis is important for relocalization of the actin cytoskeleton to the septum area

(259). These observations suggest that the *CLB1*- and *CLB2*-encoded cyclins negatively regulate the ability of Cdc28p to activate the pathways that lead to polarized actin assembly. To summarize, Cdc28p plays a key role in integrating nuclear and cytoplasmic cytoskeletal rearrangements throughout the cycle. We still do not know the phosphorylation targets of the Cdc28/Cln complex and it is likely, as discussed below, that the pathway to morphogenesis at the cortical level is much more complicated than simply direct phosphorylation by Cdc28 and that it probably involves other components such as second messengers and/or complex signal transduction pathways.

Selection of Sites of Polarized Cell Growth

Budding. An interesting aspect of cell biology is how sites of cell division are chosen. *S. cerevisiae* cells bud at defined sites on the cell surface. It was classically accepted that the site for a new bud is determined by the mating locus of the cell (*MAT*) (see reference 191 for a review) and by whether the cell was a mother or a daughter in the previous mitotic cycle. Haploid cells, either **a** or α, develop a new bud just adjacent to their previous budding site. This is termed an axial budding pattern. Diploids, in contrast, follow a bipolar pattern: **a**/α daughter cells bud at the tip of the pole opposite from their birth site (a distal pattern), although they can occasionally bud at the proximal pole as well (around or inside their birth site); mother **a**/α cells form a second bud at either pole (77a), although there is an initial preference for axial sites in new mothers (277). This behavior is under the control of the mating-type locus, since diploid **a**/**a** or α/α cells choose the axial pattern. The axial pattern has been suggested to position haploid cells close to one another for mating in wild-type homothallic strains, which can switch mating types. Diploid cells would grow distally for a more efficient colonization of the surrounding environment (166, 277).

As mentioned before, the selection of bud sites takes place in the G_1 phase after cell separation. In haploid cells, as described by Snyder et al. (406), a new Spa2p patch appears adjacent to the bud scar left from the previous cycle and the nucleus seems to rotate almost 180° until the SPB faces this new patch. A bundle of microtubules that connects the SPB with the selected point at the surface can often be detected by immunofluorescence staining. Presumably, there is a structure at the bud site that would be responsible for microtubule capture and nuclear rotation. In diploid cells, if the chosen pattern is distal, the Spa2p patch will appear in front of the SPB, which, after cytokinesis, stays just opposite the latest bud site (406). Hence, no nuclear rotation is required in distal budding. Considering this scheme, the control of cell polarity would be centered in the SPB movements toward the putative bud site in the membrane, marked by Spa2p and other proteins. However, more recent evidence provided by Chant and Pringle (77a) suggests that the determinants for bud site selection are linked to the cell surface acting as transient (in the axial pattern) or permanent (in bipolar budding) marker signals. A sensible theory is that the poles of an ellipsoidal yeast cell would be permanently marked by remnants from preferential growth at those points (74, 77a), thus explaining the basic bipolar pattern. This theory is consistent with the observation that mutations in some proteins related to the assembly of the actin cortical cytoskeleton at growth sites—Rvs167p, profilin, some actin mutants, Bem2p (see below)—cause random budding (23, 124, 174, 229). In the axial pattern, some component left from (or associated with) the 10-nm microfilaments ring could act as a membrane signal for the next bud location, since such a component might remain at the previous bud site after cytokinesis. When that mark is absent, the distal pattern is favored (146, 278, 406). Such a mark, in contrast to the bipolar determinants, is lost after starvation and refeeding (77a), and so its effectiveness is transitory. As deduced by recent studies, Bud3p is a good candidate for acting as the axial mark (74, 77) (see below).

The search for mutants with altered haploid budding patterns has identified five *BUD* genes (75, 76). This set of genes also accounts for the use of one pattern or the other. Mutations in *BUD1*, *BUD2*, and *BUD5* in haploid strains cause a random budding pattern, whereas *bud3* and *bud4* mutations lead to a bipolar pattern. When tested in diploid **a**/α strains, *bud1*, *bud2*, and *bud5* show random cell polarity while *bud3* and *bud4* do not exhibit an altered pattern. These facts strongly suggest that *BUD1*, *BUD2*, and *BUD5* are required for all sites. *BUD3* and *BUD4* are specifically required for the selection of axial sites in haploid cells. There are presumably components which are expressed only in **a** cells and α cells which are responsible for helping the selection of axial sites as well. The *SPA2* gene and the ones encoding the components of the 10-nm microfilaments ring are also essential for cell polarity, since their disruption also causes alterations in the budding pattern (146, 405). Interestingly, it has been recently reported that Bud3p colocalizes with such components, Cdc3p, Cdc10p, Cdc11p, and Cdc12p, in the area of the ring-like structure that encircles the mother bud neck (74, 77). Bud3p seems to localize to the preassembled neck filaments precisely at the time of the initiation of mitosis and remain there well after cytokinesis (77), probably accounting for the transient signal that regulates axial budding.

A search for mutants specifically affected in the bipolar budding pattern has been carried out by the team led by J. Pringle (349a), yielding *spa2*, novel alleles of *bud2* and *bud5*, and a set of four new mutants which will probably shed new light on the control of polarization in the following years. Among them, *bud6* and *bud7* mutations cause a random pattern whereas, interestingly, *bud8* mutants bud exclusively at the proximal pole and *bud9* mutants bud only at the distal pole (349a). This means that each pole may have its specific markers for budding.

BUD1, also called *RSR1*, encodes a protein homologous to known Ras proteins which are GTP-binding proteins (24, 76). GTP-bound Rsr1p/Bud1p is considered the key element for bud site selection at the poles. Rsr1p is regulated by Bud5p, which is homologous to Cdc25p, Scd25p, and Lte1p (75), a family of proteins which are thought to exchange GDP for GTP (known as GEF for GDP-GTP exchange factors), leading to the activation of Ras. *BUD2* encodes a GTPase-activating protein (GAP) that probably modulates the activation of *BUD1*/*RSR1* in vivo (338) (see the GTPase cycle in Fig. 5). Other Ras-like GTPase/GEF/GAP cycles (like the Rsr1p-Bud5p-Bud2p one) are known to exist along the secretory pathway (Ypt1p, Sec4p) (126) and in morphogenetic regulatory pathways (see below) in *S. cerevisiae.*

More recently, a novel gene involved in the development of the axial pattern has been reported and named *AXL1* (156). Remarkably, the Axl1p sequence shows homology to human insulin-degrading proteases. The fact that it is essential for the axial haploid pattern and the observation that its expression is repressed in **a**/α diploids make it a new and fundamental element for our understanding of the regulation of polarity. A putative target of this protease, Rax1p, has been cloned (cited in reference 156). Further characterization of both the function of Rax1p and the precise biological role of Bud1p/Rsr1p will help us to eventually understand the molecular controls of cell polarity.

Other mutants, different from *bud* and *spa2* mutants and those with mutations in the 10-nm microfilament ring constituents, are unable to develop a polar localization of the buds. The *PFY1*, *RVS167*, *CDC24*, and *BEM2* genes have also been reported to be essential for proper bud site selection (23, 174, 229, 404). *PFY1* codes for profilin, an actin monomer sequestering protein (174). *RVS167* encodes another putative actin-binding protein (23). Some loci functionally related to *RVS167* also show a random pattern of budding (131). It is sensible to theorize that the random budding defect observed in some of these mutants related to actin functions is simply due to a loss of the permanent signal that marks the poles of the cell, on which bipolar budding replies. Cdc24p and Bem2p (see below) are part of a complex set of regulators which are probable candidates for acting downstream from Bud1p/Rsr1p—or concurrently with it—to direct the localization of actin to the bud site, and their dysfunction prevents bud emergence (25, 229, 404, 460). Some *act1* mutant alleles and a hemizygote *ACT1*/*act1*::*LEU2* diploid also show bud site selection defects (124). Such an observation implies that actin itself must play a role, at least indirectly, in bud site selection. The effect of these mutations in bud site selection overlaps this process with the essential morphogenetic regulation pathways, making it difficult to establish a differentiation between mechanisms for the selection of polarity and mechanisms for the control of morphogenesis. This points to the existence of a strong interdependence between both processes.

In summary, analysis of the different mutants affecting budding patterns indicates that there are three different types of components that participate in bud site selection (146). There are components which mark the site of bud formation (i.e., tag components, such as the neck filament proteins, Bud3p, Bud4p, and perhaps Rax1p); there are molecules that are presumably involved in targeting components to the bud site (such as Bud1p, Bud2p, and Bud5p); and, finally, there are molecules which are targeted to the incipient bud site and assemble at that site (such as Spa2p, Cdc42p, Pfy1p, and actin). Mutations in components which mark the axial bud site lead to selection of other sites (e.g., bipolar sites), whereas mutations in targeting, early assembly, or permanent polar components can lead to the selection of random sites.

Mating projection. Growth of the shmoo projection in the presence of the opposite mating-type pheromone is also polarized (278). In this case, cell growth is polarized toward the source of the pheromone, so that the projection of each mating cell grows in the direction of its partner in order to facilitate cell fusion. The pheromone receptor at the cell surface, Ste2p (**a** cells) or Ste3p (α cells), should constitute the tag for cell wall deposition (430). Indeed, the pheromone receptor is known to accumulate at the tip of the shmoo (211) and is responsible for a cascade of events that include cell cycle arrest, a positive feedback pathway for the appearance of more receptors at the membrane, and polarized secretion of the pheromone. The fact that *spa2* mutants mate poorly and cannot form projections properly (162) strongly suggests that Spa2p, present at the projection tip, is also a component of the growth polarization machinery in the shmoo. When α-factor is uniformly distributed around *MAT***a** cells, the mating projection emerges adjacent to the bud scar, and this "axial" pattern is altered in *bud* mutants (278). This observation implies that some of the same components are probably shared in bud and mating projection site selection and emergence. Consistent with this, at least Cdc24p, Cdc42p, and Bem1p are observed in growth regions at both the bud and the shmoo projection, and *cdc24*, *bem1*, and *spa2* mutations have been isolated in a screening for mutants unable to mate with enfeebled partners (78, 80). In fact, a situation similar to bud emergence is observed in a developing shmoo. Secretory vesicles, actin, and calmodulin accumulate at the growth region, and the SPB with emanating microtubules faces the projection. As might happen during bud growth, polarized secretion of synthetic and hydrolytic cell wall enzymes to the shmoo tip is expected to be crucial for the process of projection development and cell fusion during sexual conjugation. It is interesting to speculate that the same or very similar components mediate the process of polarized secretion of cell wall components and the temporal activation of cell wall synthetic or hydrolytic enzymes in both budding and mating. This would explain the apparent lack of glucan and chitin synthases and hydrolases specifically involved in mating (Fig. 3).

Septins and Morphogenesis

The putative components of the neck ring of 10-nm filaments, Cdc3p, Cdc10p, Cdc11p, and Cdc12p, belong to a family of proteins that finds homologs in *C. albicans*, *Drosophila melanogaster*, mice, and human cells (117, 310, 315), and they have recently been designated septins (378). These proteins show an important degree of similarity, suggesting that they may perform a well-conserved function. Mutations in any of these homolog genes in *S. cerevisiae* cause a similar thermosensitive phenotype, suggesting that all these genes contribute to the assembly of the ring structure in an interdependent way (152, 173, 182, 228). Their sequences reveal a putative nucleotide-binding site (146) that may account for the regulation of their assembly. Mutants with mutations in these genes were initially isolated as defective in cytokinesis (182), but a growing body of evidence points to a broader role for the septins in morphogenetic control through the different stages of the cell cycle.

First, septins may play a role in bud site selection mechanisms, as suggested by the following observations: (i) Bud3p, a tag for axial budding as discussed above, colocalizes with these proteins (74, 77); (ii) haploid strains with mutant septins often bud at distal sites (146); and (iii) although the *SPA2* gene only seems to be essential for bud site selection and mating, *spa2* and *cdc10* mutations cause synthetic lethality (146).

Second, septins may mediate in the transduction of cell cycle Clb-dependent signals for the apical/isotropic switch, since the most characteristic phenotype of *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutations is the appearance of extremely elongated buds, resulting from an apical hyperpolarization of actin (2). The same phenomenon is observable when *CLB1* and *CLB2* genes are deleted (259).

Third, septins are assumed to play a role in cytokinesis, since (i) lack of their function causes a defect in cytokinesis and cell separation (182); (ii) their location around the septum area (152, 173, 228) suggests this possibility; and (iii) septin mutants give rise to abnormal ectopic septa (87, 403), meaning that disassembly of the ring leads to delocalization of the septum initiation process.

Fourth, septins may also control morphogenesis during shmoo formation, because Afr1p, which is involved in promoting apical growth in the shmoo, physically interacts with Cdc12p at the base of the mating projection. Additionally, the ectopic expression of the *AFR1* gene in the mitotic cycle causes a bud elongation defect like that of the septin mutations (236). These observations harmonize with the hypothesis that the cell cycle arrest caused by the presence of mating pheromone promotes a loosening of the tightly bound ring structure, thus disfavoring the formation of the neck constriction necessary for budding and therefore promoting shmoo formation.

TABLE 5. Genes related to the actin cytoskeleton

Gene (synonym)	Encoded protein	Function	Reference(s)
ACT1	Actin	Structural; essential for polarized secretion and endocytosis	124, 158, 159, 243, 315a, 319, 446[a]
ACT2 (ARP2)	Actin-like protein	Cytokinesis?	389
ACT3 (ARP3)	Actin-like protein	?	179
SAC6	Fimbrin	Actin filament bundling; essential for polarized secretion and endocytosis	3, 4, 243
SAC7	?	Essential for cytoskeleton assembly	127
MYO1	Conventional myosin heavy chain	Cytokinesis?	366
MYO2	Type V unconventional myosin	Essential for polarized secretion	171, 217, 264
MYO4	Type V unconventional myosin	?, nonessential	175
ANC1	Related to human ENL and AF-9	Transcriptional regulation?	444
ABP1	SH3 and cofilin-like domains	?, nonessential	4, 125
SLA1	SH3 domains	Important for actin assembly?	196, 261
SLA2	Talin-like	Important for actin nucleation?	196, 261
PFY1	Profilin	Actin monomer sequestering; actin assembly regulation	176, 282, 283
COF1	Cofilin	Actin severing; essential	207, 303
CAP1	Actin-capping protein α-subunit	Actin cytoskeleton assembly	4, 6, 7
CAP2	Actin-capping protein β-subunit	Actin cytoskeleton assembly	5–7
TPM1	Tropomyosin	Actin filament bundling; essential for polarized secretion	4, 122, 269, 270
TPM2	Tropomyosin	?	122
RAH3	?	Regulation of actin assembly under osmotic stress?	85
RVS167	SH3 domains	Actin cytoskeleton assembly?	23, 131
END3	Putative Ca^{2+}- and PIP2-binding protein	Important for actin assembly and endocytosis?	27
VRP1	Verprolin	Actin polarization?	119
SMY1	Kinesin-like protein	Polarized secretion?	263, 264
SAC1	?	Phosphatidylinositol metabolism?	90, 320, 448

[a] Literature on yeast actin is very extensive. Only some of the references are cited.

Fifth, septins may control the localized synthesis of the bulk of chitin by CSIII throughout the life cycle of budding yeast, since (i) loss of the 10-nm filament ring in all *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants is concomitant with the loss of the chitin ring that stands above it in the cell wall (362, 403); (ii) assembly of the neck ring occurs prior to bud emergence at the precise spot where chitin must be laid down (152, 173, 228); (iii) a *cdc10* allele, isolated in a search for thermosensitive lytic mutants, confers a less protective cell wall even at permissive temperatures (87); and (iv) at permissive temperatures, the same *cdc10* mutant forms bumpy budding scars reminiscent of those observed in *chs3* mutants (see above) (87).

In light of this record, it is tempting to speculate that the assembly of the neck filaments at the bud site may be directly or indirectly controlled by the Cdc28 kinase and that it constitutes a basic element for the control of many different morphogenetic events that govern the whole process of budding and is an interesting link between spatial chitin synthesis and cell cycle controls. The finding of other elements that interact with the septins would provide further insights into their function and regulation.

Actin Cytoskeleton: Its Role in Polarized Secretion

Actin has been shown to be essential for morphogenesis, and it also participates in other aspects of cell division, such as mitochondrial segregation and endocytosis (124, 243). As mentioned above, cell wall growth—whether in the bud site in late G_1, in the growing bud during S and G_2 phases, in the septum after mitosis, or at the shmoo tip during exposure to mating pheromone—is always accompanied by the underlying actin cortical cytoskeleton, consisting of actin patches that are easily detectable by staining fixed cells with fluorochrome-conjugated phalloidin. An interesting hypothesis, based on immunoelectron microscopy studies, has been advanced by Mulholland et al. to explain the ultrastructure of these patches (306). These authors have reported evidence that cortical actin and colocalizing proteins are distributed around thin invaginations of the plasma membrane at the sites of growth, and they propose a model in which the bottom of such invaginations is connected to actin cables. The best-characterized genes related to these structures are listed in Table 5.

ACT1 is the only gene encoding actin in budding yeasts (319), although actin-related protein genes, *ACT2* and *ACT3*, both essential, and the centractin-encoding *ACT5* have also been identified (89, 179, 305, 389). Act1p shares almost 90% sequence identity with known mammalian actins, while the other actins are more divergent. Act1p is the prevalent actin, responsible for the bulk of the cytoskeleton, and is one of the major cytoplasmic proteins in the yeast cell. Actin monomers aggregate into filaments, which constitute a basic part of certain cytoskeletal structures, such as the cytoplasmic cables and subcortical dots. Act2p (also known as Arp2, for actin-related protein) is much less abundant, and since its disruption leads to cells arrested with a large bud, like *cdc* mutations acting late in the cell cycle, it is thought to be involved in essential cytoskeletal rearrangements after mitosis. Interestingly, Act2p/Arp2p has the unique feature of carrying a consensus motif for phosphorylation by the Cdc28/cyclin complex (389). If in the future it is demonstrated that such phosphorylation really exists, this would be the first description of a direct regulatory role of Cdc28 in the temporal control of cytoskeletal events.

Strong evidence that actin is involved in different morphogenetic stages comes from analysis of phenotypes of *act1* temperature-sensitive mutants (319). *act1* mutants are often heterogeneous in size, and many large cells which contain small buds are observed. These are assumed to have arisen by polarity defects in which the mother cell grows preferentially at the expense of the bud. Other actin mutants, in which cells have elongated buds, are bumpy or multibudded, or have defects in bud site selection, have also been found (124). *act1*

mutants also exhibit a minor defect in secretion (319). These different defects are consistent with a role for actin in the polarized secretion of growth components in yeast cells.

A number of actin-binding proteins, including actin filament bundling proteins (Sac6p, Tpm1p, Tpm2p) (3, 122, 270), capping proteins (Cap1p, Cap2p) (7), monomer-sequestering proteins (Pfy1p) (174), severing proteins (Cof1p) (207, 303), and motor proteins (Myo1p, Myo2p, Myo4p) (175, 217, 366) have been identified in *S. cerevisiae*. Since the actin cortical cytoskeleton is always associated with areas of cell wall growth, we shall now consider the roles of these proteins in polarized secretion.

Some of the genes encoding putative actin-binding proteins have been cloned as suppressors of *act1* mutations (*SAC1* to *SAC7*) (3, 127, 320). Sac6p is the yeast homolog of mammalian fimbrin, and it colocalizes with both the actin cytoplasmic cables and actin dots, presumably stabilizing actin filaments (3, 123). Unexpectedly, null mutations in *SAC6* do not seem to affect viability. Another nonessential protein located at the cortical actin spots is Abp1p (for actin-binding protein), whose sequence contains domains reminiscent of those found in cofilin, a proline-rich motif, and an SH3 (Src homology 3) domain, which is characteristic of proteins involved in signal transduction pathways at the level of the cortical cytoskeleton (123, 125). Synthetic lethal mutations with *abp1* have been sought, yielding *sac6* and mutations in two novel genes, *SLA1* and *SLA2* (196). *SLA2* shows some similarity to mammalian talin genes and is indispensable for proper morphogenesis. Death is observed in *sla2 sac6* double mutants (196). The same gene was isolated by a different strategy and shown to be important for the accumulation of H^+-ATPase at the plasma membrane (309). *SLA1* is essential for the assembly of the cortical cytoskeleton and, like Abp1p, carries SH3 domains in its sequence (196). SH3 motifs are assumed to bind proline-rich regions, thus promoting protein-protein interactions that are important for the control of the actin cytoskeleton assembly in response to signalling pathways. A novel protein, verprolin, encoded by VRP1, has recently been described (119). Its main feature is the abnormally high proportion of proline found in its sequence (24%). *VRP1* (for very rich in proline) disruption leads to actin cortical cytoskeleton delocalization and aberrant cell shape (119), and hence this protein would be expected to interact with some of the SH3-containing proteins involved in morphogenetic control, such as Sla1p, Abp1p, or Bem1p.

Actin-capping proteins are also present under the cell surface growing areas (6). *CAP1* encodes for the a subunit of the yeast capping protein, while the subunit is encoded by *CAP2* (5, 7). An altered actin cytoskeleton is observed when any or both genes are disrupted, although secretion is still polarized. Mutations in the actin-capping genes are also synthetic lethal with *sac6* (4), as are mutations in two other genes, named *SLC1* and *SLC2* (225), which indeed were isolated in a screen for synthetic lethality with *cap2* mutants. The *slc2* mutation itself is also synthetic lethal with *sac6* (225). All these facts imply that the combined activity of fimbrin with talin-like, capping, and other actin-binding proteins would contribute to the integrity of the actin cortical cytoskeleton.

Also colocalized with actin in regions of active cell wall growth is the *COF1* gene product, a homolog of the mammalian actin-severing protein, cofilin (207, 303). The yeast cofilin gene, unlike yeast fimbrin and talin-like protein, is essential. Another ubiquitous actin-binding protein is profilin, an actin monomer-sequestering protein presumably involved in cortical cytoskeleton assembly. Yeast profilin is encoded by *PFY1*, a gene whose absence has a dramatic effect on cell growth and chitin distribution (174, 282, 283). The binding of profilin to actin is antagonized by phosphatidylinositol-4,5-biphosphate, suggesting that changes in membrane phospholipids may be an important regulatory pathway leading to subcortical actin polymerization (275). Supporting this hypothesis, Cap2 function is inhibited by phosphatidylinositides (6) and mammalian cofilin has also been reported to bind phospholipids (455). It is likely that profilin, and perhaps cofilin and the capping protein, could have a role in regulating actin polymerization in response to changes in membrane phospholipids. Additional evidence pointing to the influence of inositol glycerophospholipids on the actin cytoskeleton comes from one of the aforementioned actin suppressors, *SAC1*, which does not encode an actin-binding protein, as expected, but, rather, encodes a Golgi membrane protein presumably involved in phosphatidylinositol metabolism (90, 448). Further studies must be carried out to assess the role that each protein plays in these interactions.

A gene involved in the endocytic process, *END3*, has also been reported to be essential for actin polarization. The encoded protein contains consensus sequences for the binding of Ca^{2+} and phosphatidylinositol-4,5-biphosphate (27). Actin and fimbrin themselves are important for vesicle internalization during endocytosis in yeast (243). Since disruption of the *END3* gene also affects both actin distribution and chitin deposition (27), this gene may have a function in polarized growth.

A screen for mutations that preclude complementation of a thermosensitive *act1* allele, carried out by the research team led by D. G. Drubin, yielded the four *anc* (for actin-noncomplementing) mutations (445). The *ANC1* gene has been cloned and characterized. Anc1p is a nuclear protein showing homology to human proteins involved in the development of leukemias. Deletion in *ANC1* and *SLA1* leads to synthetic lethality, but, interestingly, the *anc1* deletion phenotype alone causes a unique pattern of cytoskeletal and morphogenetic alterations (444). One of the unique features of an *anc1* mutant is the delocalization of Spa2p. How this protein influences actin and Spa2p polarization remains obscure, although it would open an unexplored field in the control of morphogenesis.

Yeast unconventional class V myosins are encoded by the *MYO2* and *MYO4* genes. Mutations in *MYO2* lead to severe defects in polarized secretion and morphogenesis (171, 175, 217). Temperature-sensitive mutants fail to form a bud, and null mutants are nonviable. The actin-Myo2p interaction in the yeast cell is thought to promote the transport of secretory vesicles along actin cables (2, 171, 227), although recent data have shown that its cellular localization matches that of cortical actin (264). Its homolog *MYO4* is not essential (175), and so far there is no evidence for its involvement in polarized secretion. A conventional type II myosin heavy chain, the *MYO1* gene product (366, 416), also shows morphologic alterations when mutated, reminiscent of those observed when *MYO2* or *MYO4* is overexpressed (175). However, its function is unknown, and it seems unlikely that it would play a role in bud development.

A component which interacts functionally with actin cables, tropomyosin (encoded by the *TPM1* gene), has also been identified (269, 270). Disruption of *TPM1* leads to abnormal chitin distribution but does not cause a very dramatic effect in cell development. The accumulation of secretory vesicles observed in *tpm1* mutants is diminished when earlier steps of the secretory pathway are affected (*sec13* and *sec18* mutations) (270), suggesting that the role of tropomyosin in secretion takes place at a post-Golgi stage. A conditional mutation in *MYO2* is synthetic lethal with *tpm1* (270), and its pattern of vesicle accumulation also suggests a post-Golgi role in the secretory pathway (171). A minor nonredundant tropomyosin, encoded by *TPM2*, is found in *S. cerevisiae* (122). A *tpm2* disruption

exhibits no perceptible morphogenetic alterations, but, interestingly, overexpression of the gene causes a switch from axial to bipolar budding. Since this phenotype is remediable by cooverexpression of *TPM1* and since the two tropomyosins affect each other's binding to actin in vitro (122), it is likely that at least Tpm1p interacts with the bud site assembly mechanisms.

To summarize, actin, unconventional myosin, and tropomyosin are important for both the establishment and development of polarized secretion. It is conceivable that other genes involved in the general secretory pathways might also be important for cell surface growth. Study of the *SEC* genes (322), especially those involved in the late stages of secretion, might also provide important clues for our understanding of how determinants for cell wall growth are targeted to discrete points on the cell surface. For instance, it has been reported that a membrane complex formed by the proteins Sec8p and Sec15p, probably under the control of the small GTP-binding protein Sec4p, is responsible for exocytosis of the secretory vesicles (40), a process that would obviously be critical for the release of cell wall precursors to the periplasmic space. Consistent with this, Sec4p accumulates at sites of cell growth (321). Nevertheless, the secretion of invertase is not affected in either *tpm1* or *myo2* mutants (171, 270), implying the existence of two distinct secretory pathways: an actin-dependent one involved in cell surface growth, and a nonspecialized one which is not dependent on actin.

Defects in the actin cytoskeleton also lead to osmosensitivity (319). In fact, an actin mutant has been isolated in a screen for osmotically fragile mutants (386). Considering the data which we have reviewed so far, a failure in the actin cytoskeleton would lead to impaired polarized secretion and therefore to a defective cell wall unable to efficiently protect the cell from an anisoosmolar environment. Strikingly, however, after exposure of a wild-type strain to hyperosmotic medium, the actin cytoskeleton disassembles for a time and then reorganizes again (85, 260a), suggesting that some factors, specifically present under osmotic stress conditions, might account for this rearrangement. Chowdhury et al. (85) have isolated three suppressors of the osmosensitive (but not the thermosensitive) defect in *act1-1* conditional mutants. One of these genes, *RAH3*, is a new putative actin-binding protein. Its disruption leads to an altered actin localization and chitin distribution around the cell surface (85). This phenotype is common to previously described mutations in many other genes that code for actin-associated proteins. Also supporting a specific control of actin organization in response to environmental stress, another mutation which leads to abnormal morphology, impaired actin distribution, and altered budding patterns lies in the *RVS167* gene. *RVS167* is predicted to encode a protein with an SH3 domain; the gene was originally identified in a screen to search for genes essential for the maintenance of viability during the stationary phase (23). Other genes related to the function of *RVS167*, namely, *RVS161*, *SUR4*, and *FEN1*, have been reported to display altered budding patterns (131), probably because of the same involvement in cortical actin rearrangements.

Microtubules Are Not Required for Morphogenesis

Nocodozole-treated cells and *tub2* (tubulin-deficient) mutants form buds and mate normally (203, 212). Thus, microtubules are not required for bud or mating projection formation. However, it is worth mentioning two facts that suggest that microtubules may play an ancillary role in bud formation: (i) as stated above, microtubules extend into the nascent bud and shmoo tip, and the SPB (the structure which organizes the microtubular cytoskeleton) always faces the growing areas (2, 227); and (ii) strains bearing a temperature-sensitive mutation in *myo2* can be suppressed by overexpression of *SMY1*, a gene encoding a kinesin-related protein (263). Smy1p has been shown to colocalize with Myo2p at the regions of polarized growth instead of displaying the localization pattern of microtubules (264). Thus, although tubulin is dispensable for morphogenesis, we cannot presently discard the possibility that actin and microtubules participate in polarized cell growth in yeast cells, with actin playing the most predominant and essential role.

Role for Ca^{2+}?

As pointed out above, such an accurate localization of the cytoskeletal elements to the bud site must be strictly controlled, and this control must somehow be connected to that of the nuclear events of the cell cycle. We have already discussed the putative leading role of the *CDC28* gene product, but other pieces of the puzzle remain to be deciphered. A significant key to the morphogenetic machinery is *CDC24* (Fig. 5; Table 4) (see below) (297, 404). Mutants defective in this gene fail to organize all the cytoskeletal elements for polarized growth (1), causing the cells to develop large, unbudded morphologies with multiple nuclei (404). *CDC24* encodes a putative calcium-binding protein (298). In fact, some *cdc24* mutations elicit the inability to bud in a calcium-dependent way (329). Besides, the sequence of the aforementioned End3p, involved in actin function, also shows a putative calcium-binding domain (27). These observations suggest an interconnection between calcium intracellular levels and morphogenetic events in yeast cells, thus establishing a correlation with cell surface events in higher eukaryotes, in which free Ca^{2+}, together with changes in phospholipids and phosphorylation, is believed to regulate actin rearrangements in response to different stimuli (410).

It is worth mentioning that Hkr1p, a transmembrane protein that may control 1,3-β-glucan synthesis through an unknown mechanism (see above), also bears a consensus sequence for calcium binding (248). Moreover, as stated in a previous section, the *FKS1* and *FKS2* genes, believed to encode subunits of the 1,3-β-glucan synthase, might be regulated by Ca^{2+}/calmodulin-dependent calcineurin (120, 151). The possibility therefore exists that the regulation of glucan synthesis is also mediated by the levels of free Ca^{2+}.

The isolation of *MID1* in a screen for cells that lyse after differentiating into shmoos (206) sheds some light on the role of calcium in morphogenesis during mating. *MID1* is predicted to encode a plasma membrane protein which is important for Ca^{2+} uptake. In fact, lysis of mating forms in *mid1* mutants is prevented when high concentrations of $CaCl_2$, but not other salts, are added to the medium (206). Furthermore, a second mating pheromone-induced death gene, *MID2*, has been cloned and seems to encode another membrane protein, in this case showing a putative calcium-binding domain (cited in reference 206).

Additional evidence supporting a presumptive role of calcium ions in the morphogenetic process is the presence of calmodulin (Cmd1p) in active growth sites (43). Like actin, calmodulin localizes to the selected bud site before bud emergence; it then passes to the tip of the growing bud and, finally, redistributes to the septum at the time of cytokinesis. Furthermore, actin and calmodulin localizations are interdependent (*cmd1* mutants fail to localize actin, and *act1* mutants fail to localize calmodulin) (43), and a subset of *cmd1* mutants fail to polarize cell surface growth in a way reminiscent of *cdc24*

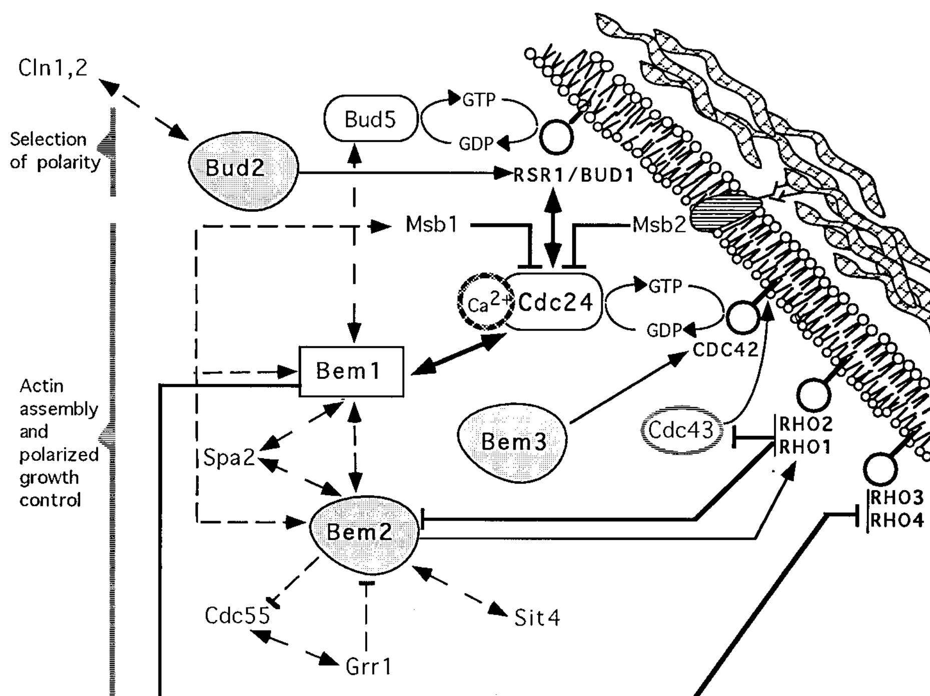


FIG. 5. Genetic and functional interactions of the small GTPases of the Ras superfamily that control cell polarity and polarized growth. Symbols: ○, small GTPases; ▭, GEFs; ◯, GAPs; ◯, GGTase; —|, multicopy suppression; – – –|, mutational suppression; ↔, physical interaction; ◄– – ►, synthetic lethality; →, functional interaction.

mutants (327). Nevertheless, polarized calmodulin localization does not depend on its ability to bind Ca^{2+} since it persists when the cation-binding site is mutated (43). Although it is very unlikely that actin and calmodulin would interact physically, it is plausible that calmodulin might interact with any actin-binding protein. Actually, a candidate may be Myo2p, which also colocalizes with actin (264) and bears in its sequence six putative calmodulin-binding sites (217). *cdc24* mutants fail to localize both calmodulin and actin (413), so this gene must direct a crucial step in the control of the rearrangements taking place prior to bud emergence.

To learn how *CDC24* manages to regulate morphogenesis, researchers have attempted to find genes that could interact with it functionally (see Fig. 5). Multicopy suppressors of *cdc24* mutations are found in *BUD1/RSR1*, *CDC42*, *MSB1*, and *MSB2* (24). The isolation of *BUD1/RSR1* (see above) links the events that regulate bud site selection to the essential events required for polarized growth. *CDC42* encodes a Ras-like GTPase (see the following section), which exhibits a *cdc24*-like phenotype when disrupted (1). *MSB2* encodes a putative transmembrane protein lacking any known catalytic or regulatory activity; its abundance in Ser/Thr residues, an uncommon feature also found in chitinase, Kre1p, Hkr1p, Fus1p, and some other mannoproteins, suggests that it could be O glycosylated (26). Both *MSB* (for multicopy suppression of budding defect) genes show no phenotype when disrupted, and a synthetic lethal screen was devised for uncovering *MSB1*-related or interacting genes (25). These experiments yielded two genes whose defect also results in a *cdc24*-like phenotype: large unbudded multinucleate cells. They were called *BEM1* and *BEM2* (for bud emergence). Interestingly, *BEM1* is important for cell polarity in both bud and shmoo development and contains two SH3 domains in its sequence (79, 80), like those in Sla1, Abp1, Rvs167 (23, 125, 196). It has recently been reported that Bem1p and Cdc24p interact physically (345). Moreover, Ca^{2+} seems to inhibit such interaction in vitro (459). All the evidence suggests that Bem1p and Cdc24p are key components required for the attachment of the actin cytoskeleton to the cell surface and that calcium may mediate in their function.

Rho Proteins

A growing collection of membrane-associated Ras-related GTP-binding proteins have been found to participate in the control of morphogenetic processes (Fig. 5). They belong to the Rho group of proteins within the Ras superfamily. The common features of Rho proteins are that (i) they all carry prenylation sequences, and the addition of lipid moieties to these domains modulates their binding to membranes and may therefore affect their activity; (ii) like Ras, they all bind guanine nucleotides, being active when bound to GTP and inactive when bound to GDP; (iii) they are able to hydrolyze GTP, for which they require a GAP, which acts as a negative modulator; (iv) a GEF is involved in their activation, closing the GTPase cycle; and (v) they are thought to stimulate actin reorganization in vivo (168; see reference 177 for a review).

We currently know of five Ras-like-encoding genes in budding yeast which are involved in morphogenesis: *CDC42*, *RHO1*, *RHO2*, *RHO3*, and *RHO4* (1, 216, 276, 290, 351). *CDC42* is by far the best characterized of them. The structure and functionality of Cdc42p seem to be highly conserved, since homologous genes playing an identical role have been found in the fission yeast *S. pombe* (296) and even in human cells (177). Supporting its role in actin polarization, it has been recently shown that Cdc42p is essential for the GTP-dependent nucleation of actin in fixed yeast cells (261). As mentioned before, *CDC42* is known to act as a multicopy suppressor of *cdc24* mutations. Recently, Zheng et al. reported that Cdc24p is indeed a quite specific GEF for Cdc42p, while a novel gene, designated *BEM3*, which had been isolated as a suppressor of

bem2 mutations, codes for a dispensable but also very specific GAP that completes the GTPase cycle of the *CDC42* gene product (460, 461) (Fig. 5). To be added to the list headed by SH2 and SH3 domains involved in protein-protein interactions in regulatory pathways at cortical level in eukaryotes (233, 341) is a recently described kind of domain present in undermembrane signalling proteins; it has been called PH (for pleckstrin homology) (307). Interestingly, the amino acid sequences of both Cdc42p modulators, Cdc24p and Bem3p, reveal a PH domain.

Mutations in the *BEM2* gene have been isolated in different approaches: (i) as synthetic lethal with *msb1* (25); (ii) in a different screen for synthetic lethality with *spa2* (279); (iii) in a third synthetic lethal screen with *bem1* (345); (iv) in a search for mutations suppressing *cdc55* (see below) (187); (v) in a screen for increase-in-ploidy mutants (73); and (vi) in a screen for lytic mutants (87). Bem2p is another Rho-GAP but is unable to function in the Cdc42 GTPase cycle (345, 460, 461). Experimental evidence has shown that Bem2p would rather act as a GAP for Rho1p or perhaps Rho2p GTPases (229, 345). *bem2* mutations cause a budding defect similar to that observed in *cdc42* and *cdc24* mutants, although less severe. Cell enlargement is severe at 37°C and is accompanied by actin and chitin delocalization, appearance of huge bud scars, and defects in cell separation (87, 229, 345). The cell wall composition in *bem2* mutants may be altered, since they are more sensitive than controls to killer toxin, cell wall synthesis inhibitors, and exogenous enzymatic degradation of β-glucan (87). The observed increase in ploidy is probably due to consecutive nuclear divisions uncoupled from budding. Synthetic lethality and mutational suppression experiments performed on *bem2* (Fig. 5) suggest that phosphorylation could be involved on its regulation (229). Taken together, the above observations support the hypothesis that cell cycle-controlled polarization of growth and cell wall biogenesis are interdependent or, at least, obey common signals.

cdc24 suppression by *BUD1/RSR1* (24) could be due to overlapping substrate specificity or functional redundancy among the different Ras-like proteins, although experimental findings favor the hypothesis that Cdc24p would establish a close relationship between the control of bud development and the selection of cell polarity. In fact, it has recently been reported that GTP-bound Rsr1p interacts in vitro with Cdc24p (459). This interaction does not influence the GEF function of Cdc24p on Cdc42p, and it appears to stabilize the GTP-bound form of Rsr1p. It is tempting to theorize that a sequential activation of both GTPase cycles would be mediated by Cdc24p. Another clue to the link between bud site selection and bud emergence controlling sets is the fact that the triple mutant *cln1 cln2 bud2* shows an unbudded *cdc24*-like phenotype instead of the expected plain defect in budding pattern (28, 101). The same lethal effect is caused by an activated *RSR1* allele on a *cln1 cln2* background (28); this is logical if one considers that Bud2p is a GAP and thus a negative regulator for Rsr1p (338). A third link is given by the fact that a functional interaction may occur between *BUD5* (the GEF for Rsr1p) and *BEM1* based on the synthetic lethality caused by their concurrent mutations (75). The hypothesis that Cdc42p activation ultimately depends on G_1 cyclins is attractive. Another interesting hypothesis is that the SH3 domains in some actin-binding proteins would be interacting with proline-rich regions of the Rho GAPs or exchange factors (86). PH domains, such the ones in Cdc24p and Bem3p, might themselves interact with small GTP-binding proteins (307). The resulting complex would trigger actin polymerization at the presumptive bud site.

Regarding the prenylation of these proteins, two kinds of enzymes are responsible for that function: farnesyltransferases (FTase) and geranylgeranyltransferases (GGTase). *CDC43/CAL1* is known to encode the β-subunit of a GGTase essential for Cdc42 function (1, 328, 330), whereas a putative α-subunit, which may work equally for both FTase and GGTase functions, is encoded by *RAM2* (186, 292). *RHO1* and *RHO2* were in fact isolated in a search for suppressors of a *cdc43* mutation (351). Both are prenylation targets for the *CDC43*-encoded GGTase (330) and may also be involved in the control of the cortical actin network. In fact, Bem2p has been shown to possess specific GAP activity over Rho1p, at least in vitro (345, 460), and Rho1p has been immunolocalized at the areas of polarized growth (452).

Finally, the family of yeast morphogenesis-related Rho proteins (see Fig. 5 for a comprehensive view) includes two more recently identified genes, *RHO3* and *RHO4* (290). Their defect causes osmoremedial cell lysis, which is suppressed by *CDC42* and *BEM1* (291). Suppressors of the *rho3* mutation have been isolated (291) and should probably give further clues to how these proteins manage to control the organization and attachment of the actin cytoskeletal apparatus to a polarized site under the cell surface.

Phosphorylation, Cell Integrity, and Morphogenesis

The recent identification of phosphorylation pathways essential for cell integrity has increased the number of known morphogenetic controls. In a relatively short time, many regulatory genes coding for protein kinases have been cloned and characterized and their corresponding products have been shown to be organized in a conserved structure typical of the mitogen-activated protein (MAP) kinase signalling pathways (192). The functionality of two of these cascades—namely, the *PKC1*-controlled pathway (252) (Fig. 6) and the high-osmolarity glycerol pathway (40)—is important for the generation of an osmotically stable cell wall structure and therefore for morphogenesis. We shall refer to the former as the cell integrity/proliferation pathway and to the latter as the osmosensing pathway (Fig. 7). Both molecular and biochemical approaches have led to the identification of most of the components of these pathways. Their homology to the members of the well-known pheromone response pathway (Fig. 7) has been of great help in the definition of their role and properties. A description of some of the experimental observations reported thus far should help us to define a perspective for the precise role of these phosphorylation pathways in the control of morphogenesis. Nevertheless, the field is fast moving, and we can expect that much more information will have to be gained before we have a complete picture of the role of the cascades, the mechanisms that activate them, and the kind of responses they produce. A list of protein kinases and phosphatases presumably involved in morphogenesis is given in Table 6.

The *PKC1*-controlled pathway is essential for the integrity of proliferating cells. The integrity of proliferating yeast cells depends on the functionality of a number of protein kinase genes whose products seem to act sequentially. These are *PKC1*, predicted to encode the only type C protein kinase so far detected in yeast cells, and a three-gene module characteristic of the extracellularly regulated kinase pathways, consisting of a MAP kinase kinase kinase (MEKK), a MAP kinase kinase (MEK), and a MAP kinase component (Fig. 7). In this case, the corresponding genes are *BCK1/SLK1* (encoding the MEKK), *MKK1* and *MKK2* (redundant in their function and encoding MEK), and *SLT2/MPK1* (encoding the MAP kinase). Strains with null and other types of mutations in these genes

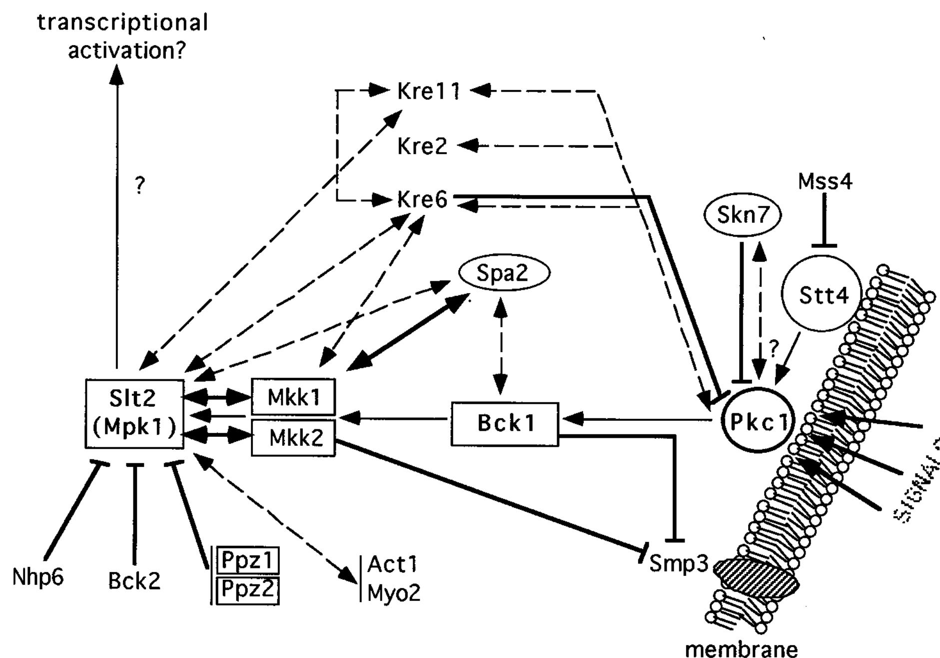


FIG. 6. Genetic and functional interactions of the members of the cell integrity/proliferation phosphorylation cascade. Symbols: →, functional interaction; ◄- - ►, synthetic lethality; —|, multicopy suppression; ↔, physical interaction.

display a thermosensitive lethal phenotype due to lysis of growing cells, which can be prevented by osmotic stabilization of the corresponding medium. This supports the notion that the functionality of these genes would be required for the generation of a stable cell wall structure. However, the corresponding genes were isolated by different strategies. The functional and genetic interactions among these genes are shown in Fig. 6.

The family of mammalian serine/threonine protein kinases C is known to be vital in various types of cells, participating in the transmission of extracellular signals for the control of cell growth and proliferation (326). The *PKC1* gene was cloned from *S. cerevisiae* on the basis of its homology with probes derived from the catalytic and regulatory domains of rat *PKC* genes (258). Deletion of the *PKC1* gene in *S. cerevisiae* leads to death, with a terminal phenotype characterized by an arrest in growth at the stage of the cell cycle where a small bud has been formed and the DNA has been replicated. Therefore, *PKC1* function would be essential for the G_2-to-M transition. However, the significance of *PKC1* in cell wall studies was advanced when it was demonstrated that *pkc1* strains are osmotic remedial (256). Furthermore, the *PKC1* gene was reisolated by complementation of *S. cerevisiae cly15* mutants, which are autolytic and can grow only in osmotically stabilized medium (337). A thorough characterization of *pkc1* deletion strains proved that Pkc1p function is essential for the formation of an osmotically stable cell wall (257, 337). The morphology of cell wall layers in ultrathin sections is somewhat modified in the strains lacking the gene (337). Cell growth arrest is accompanied by an arrest in protein synthesis; viability is lost, and the cytoplasmic contents are released to the external medium (256).

The *PKC1* gene was also isolated by a third strategy, based on a screen for staurosporine-sensitive mutants, this compound being a specific inhibitor of type C protein kinases (456). The same strategy has also led to the isolation of two morphogenesis-related protein kinases C in the fission yeast *S. pombe* (421). Yoshida et al. have recently reported that as a result of the same staurosporine-based screening, a second gene, *STT4*, determines sensitivity to this drug and, interestingly, seems to participate in the same pathway as *PKC1* (457). This conclusion was deduced from the fact that the staurosporine sensitivity of *stt4* mutants was suppressed by *PKC1* overexpression. Furthermore, *stt4* deletion strains also show an osmoremedial phenotype. *STT4* encodes a phosphatidylinositol 4-kinase (457). Phosphatidylinositol kinases are often involved in signalling pathways in mammalian cells.

A potential downstream protein kinase gene, *BCK1* (for bypass of C kinase, also called *SLK1* and *SSP31*), was identified as an extragenic suppressor of the *pkc1* mutant phenotype (253). The mutant allele displays the mutation upstream from the catalytic domain, close to a potential protein kinase C phosphorylation target. *pkc1* mutations are suppressed by the mutant allele of *BCK1* but not by overexpression of wild-type Bck1p (253). *BCK1* was also isolated by two other strategies: (i) synthetic lethality with the mutant allele *spa2* (97) (as mentioned above, Spa2p localizes to positions of polarized growth and is involved in polarization during budding and mating [80, 162, 405]); and (ii) high-copy suppression of *smp3* mutants (208). Interestingly, the *SMP3* gene encodes a transmembrane protein whose defect causes defects in cell growth and plasmid stability (208, 209).

Deletion of *BCK1* also leads to a thermosensitive lytic phenotype, which is rescued by addition of osmotic stabilizers (e.g., sorbitol) to the growth medium (253). Consistent with the *spa2* synthetic lethal phenotype, *BCK1* is also important in cell morphogenesis; *bck1* mutants are often heterogeneous in size and shape, and they exhibit a shmoo formation defect (97). Diploid cells homozygous for the *slk1* trait display sporulation deficiency and poor viability under conditions of nutrient deprivation, thus suggesting a role for *BCK1/SLK1* in the transitions from mitotic cycle to meiosis or stationary phase (97, 99).

The cloning of protein kinase genes relevant for cell integrity continued with the isolation of other multicopy suppressors of the phenotype of *smp3* mutants, which identified *MKK1* (210).

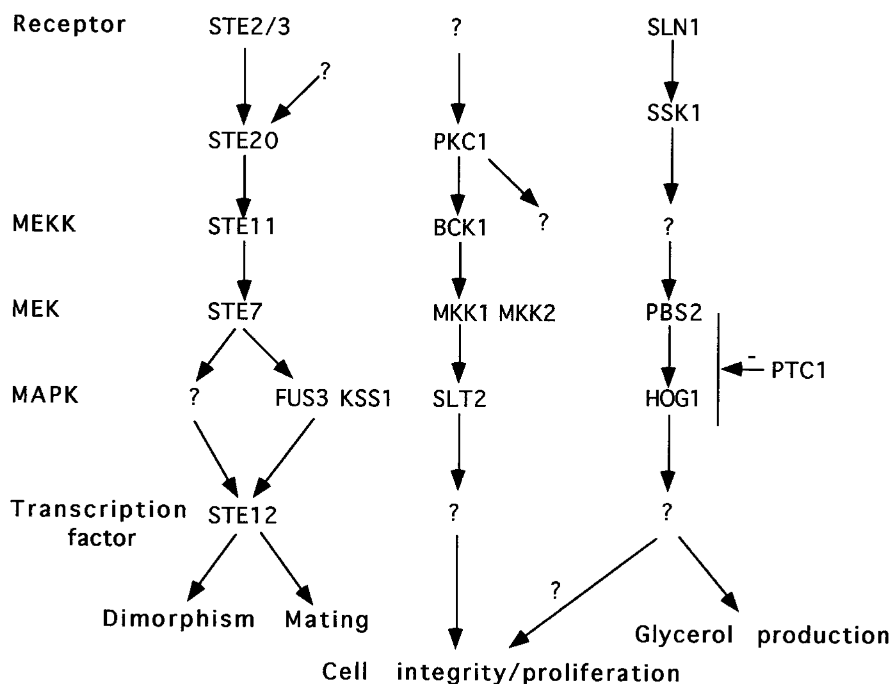


FIG. 7. Three signalling cascades in which MAP kinases are involved in *S. cerevisiae*: the mating pheromone response pathway, the cell integrity/proliferation pathway led by Pkc1, and the osmosensing pathway.

In this case, cells deleted in the *MKK1* gene do not display any altered phenotype, because there is a very homologous redundant gene, *MKK2*, which was isolated on the basis of that homology. The double deletion of both *MKK1* and *MKK2* does lead to cells that are nonviable at 37°C and display an autolytic phenotype similar to the one described for other kinase mutants (210).

The discovery of the MAP kinase component of the cell integrity/proliferation pathway was related from the beginning to cell wall research, since its isolation was made possible by the use of thermosensitive autolytic mutants (62), which had been conceived as strains with potential alterations in their cell wall assembly (317). The search for a DNA fragment that complements the lytic phenotype of *S. cerevisiae lyt2* mutant strains led to the rather unexpected isolation of a protein kinase gene, *SLT2*/*MPK1* (422), that was clearly involved in these phenomena. Although initial genetic evidence pointed out that *SLT2* was a suppressor of the altered phenotype in *lyt2* strains, the recently rescued original *lyt2* mutant allele proved to be an *slt2* mutant (70). Deletion of the *SLT2* gene resulted in a thermosensitive phenotype of cell lysis at 37°C with the corresponding release of the intracellular contents, which could be rescued under osmotic stabilization conditions (107, 422). Lysis requires active growth and takes place in cells with small buds. Interestingly, other phenotypes of the *slt2* mutant include caffeine sensitivity, failure to undergo cell cycle arrest under nutrient depletion conditions, accumulation of secretory vesicles, and delocalization of chitin and actin in the mitotic cycle (99, 252, 293).

Slt2p, the putative product of the *SLT2* gene, is a homolog of serine/threonine protein kinases, with a significant similarity to other yeast protein kinases such as Fus3p and Kss1p (422)

TABLE 6. Protein kinases and phosphatases related to morphogenesis

Gene	Encoded protein	Function[a]	Reference(s)
PKC1	Protein kinase C	Osmoremedial cell lysis	256, 258, 337
BCK1 (*SLK1*, *SSP31*)	MEKK	Ts^- cell lysis, (osmoremedial), mating defect	97, 99, 208, 253
MKK1, *MKK2*	MEKs	Ts^- cell lysis (osmoremedial, both disrupted)	210, 407
SLT2 (*MPK1*)	MAP kinase	Ts^- cell lysis (osmoremedial)	252, 289, 293, 407, 422
PPZ1, *PPZ2*	Protein phosphatases	Ts^- cell lysis (osmoremedial, both disrupted)	103, 204, 251, 348
PBS2	MEK	Defective osmoregulatory response; polymyxin sensitivity	34, 35, 40, 214
HOG1	MAP kinase	Defective osmoregulatory response	40
PTC1	Protein phosphatase	?	214, 280
PPH21, *PPH22*	PP2A (catalytic subunits)	Morphogenetic defects; arrest in G_2	264a, 373
PPH3	PP2A-like	Together with *pph21* and *pph22*, growth defect	373
TPD3	PP2A (regulatory subunit A)	Cs^-; morphogenetic defects	434
CDC55	PP2A (regulatory subunit B)	Cs^-; morphogenetic defects	187, 229
YCK1, *YCK2*	Casein kinase I	Ts^-; morphogenetic defects	364
SPS1	Protein kinase Ste20 homolog	Abnormal spore wall formation	155
SMK1	MAP kinase	Abnormal spore wall formation	241

[a] Ts^-, thermosensitive; Cs^-, cold sensitive.

and, in general terms, with MAP kinases known to be part of cascades regulated by external stimuli that are transmitted through appropriate receptors. The gene was placed in the cell integrity/proliferation pathway and renamed *MPK1* when it was reisolated as a multicopy suppressor of a *bck1* defect (252). The functionality of Slt2p is dependent on the Lys-54 residue, the putative active center for ATP binding (289), as well as on Thr-190, a potential phosphorylation site conserved in MAP kinases and critical for their regulation (252). Residue Tyr-192, another potential phosphorylation site of importance in the regulation of these proteins, was shown to be only partially needed for the functionality of the enzyme (252), since the gene did not completely lose functionality upon mutation of this residue. Another salient and intriguing feature of the protein is a polyglutamine fragment, consisting of 16 residues of this amino acid close to the C-terminal end, whose deletion does not seem to affect the functionality of the protein (346).

Mechanisms of activation and signal transduction in the cell integrity/proliferation pathway. Epistasis relationships among the above-mentioned protein kinase genes have been useful in establishing their order in the cell integrity/proliferation pathway. However, the biochemical analysis of their interactions, as well as the activation of specific catalytic activities, is providing new insights in the characterization of this pathway. This information should eventually lead to the clarification of two major issues with regard to the cell integrity/proliferation pathway that are still unknown. These are the nature of the stimulus and the sensor that receives it, thus activating Pkc1p, and the specific functions downstream of the MAP kinase encoded by *SLT2*, which are activated as a result of the transmission of the signal. Pkc1p phosphorylates substrates similar to those of its mammalian counterpart and strongly associates with particulate fractions. Nevertheless, its in vitro activity does not seem to be regulated by typical cofactors such as phospholipids, diacylglycerol, and Ca^{2+}, so that it appears as a distantly related member of the protein kinase C superfamily (8, 440).

Many pieces of evidence are confirming the proposed sequence of reactions between the protein kinase components of the cascade. For example, biochemical evidence supporting the activation of Bck1p by Pkc1p has been obtained (255), and it has also been demonstrated that Slt2p interacts both in vivo and in vitro with both Mkk1p and Mkk2p (407).

The thermosensitivity of most of the *S. cerevisiae* null mutants with mutations in genes of this pathway was somewhat intriguing at the beginning. It was difficult to explain why these genes are required only for growth at 37°C when the functions they affect, i.e., the generation of an osmotically stable wall structure, should be required at any temperature. Very recent data obtained in experiments in which the cells were subjected to thermal shocks point to the critical requirement of a functional cell integrity/proliferation pathway at elevated temperatures. These data have been obtained in the course of studies with *SLT2* and *MKC1*, the *C. albicans* homolog of *SLT2* that displays more than 50% identity with its *S. cerevisiae* counterpart (312). Double-deletion strains *mkc1/mkc1* of the opportunistic diploid fungus were shown to give rise to a cell wall that was more sensitive to the attack of exogenous lytic enzymes than wild-type cell walls were. However, these changes seem to have an effect mostly on the stability of the cells to thermal shocks at 55°C, the null mutant displaying a clear loss of viability that affected only growing cells and not stationary-phase cells (312). These data are very consistent with those of Levin (255), who observed a protein synthesis-independent activation of the phosphorylation capacity of Slt2p by pretreatment at different elevated temperatures. The activation was clearly dependent on the temperature and occurred only in growing cells in which *PKC1* and *BCK1* genes were functional.

Watanabe et al. (440) favor the idea that another, as yet unknown protein kinase must control the activity of Pkc1p. The discovery of this enzyme should be of considerable help in the identification of the sensor that activates Pkc1p to transmit the corresponding signal through the cell integrity/proliferation pathway. The consideration of the different types of stimuli that seem to activate a response in the cascade should also prompt adequate hypotheses and experimental strategies for the elucidation of its precise role. As stated above, heat shocks clearly activate signal transduction in this pathway, and Levin (255) has suggested that the alterations in membrane fluidity as a result of the temperature shift would activate the cascade in a response aimed at adapting membrane composition to the new situation. In any case, when considering the nature of the stimuli affecting the sensor of the Pkc1p-controlled pathway, we cannot forget other evidence that points to at least two other types of potential inputs in the cascade (see below). One may be defined by a possible relationship with the Cdc28p kinase (293), and the other input would be produced under low-osmolarity conditions (172). A potential upstream modulator of the cascade lies, as stated above, in the Stt4p phosphatidylinositol kinase.

Whether it is an alteration in membrane fluidity/composition and/or the wall structure or a different stimulus that triggers the sensor of the cell integrity/proliferation pathway, it is clear that the study of the functions downstream of Slt2p should also be of much help in this regard. Other MAP kinase pathways, including the *S. cerevisiae* mating/dimorphism response, converge on transcription factors (Fig. 7) (192). Many *slt2* and *bck1* defects are suppressed by overexpression of a set of chromatin proteins, Nhp6Ap and Nhp6Bp (98). These proteins are probably not direct substrates of Slt2p/Mpk1p, because they are not phosphoproteins (98). Instead, a likely model is that Nhp6Ap and Nhp6Bp function with a transcription factor which is a substrate for Slt2p. Studies in progress in different laboratories indicate that transcription factors are under the control of the cell integrity/proliferation pathway.

There is evidence for a potential involvement of Pkc1p in cell wall-related functions other than the ones it can affect through the kinase cascade. The basic fact is that while *pkc1* mutants require an osmotically stabilized medium for viability at any temperature (256, 337), mutants with mutations in the downstream kinase genes of the cascade display this phenotype only at the nonpermissive temperature (210, 252, 422). This indicates that *PKC1* must be involved in cell integrity through a bifurcated pathway (257); Pkc1p could affect cell wall functions not only through its activation of the phosphorylation cascade but also by direct phosphorylation of some of the enzymes involved in the biosynthesis of cell wall structural components. This view has been reinforced by recent findings (47, 369) that showed suppression of the lytic phenotype of *pkc1* mutants by overexpression of the product of the *KRE6* and *SKN7* genes, which are involved in the synthesis of 1,6-β-glucan (Fig. 6).

Osmosensing pathway and its relationship with the cell integrity/proliferation pathway. Recent evidence also points to the existence of yet another set of regulatory genes with a critical function for the stability of the cell, in this case under other stress conditions such as the increase in external osmolarity. Eukaryotic cells adjust internal osmolarity to osmotic changes in the external medium (284) by creating an osmotic gradient through the plasma membrane that allows water into the cell. In *S. cerevisiae*, an increase in external osmolarity leads to a stimulation of the synthesis of glycerol and a decrease in

the permeability for this molecule (33). Of most interest, in the context of the transduction of signals relevant for cell integrity, is the identification of genetic elements that seem to define another cascade of kinases that would determine the cellular osmoregulatory response. This osmosensing system is clearly defined by the isolation of mutants that fail to implement the osmoregulatory response. To a certain extent, these mutants would be the opposite of the autolytic mutants discussed above, since they are unstable at high external osmolarities. Molecular approaches to the characterization of these mutants (40) have led to the isolation of *HOG1*, coding for a MAP kinase component in this pathway, and to the reisolation of *HOG4*, previously known as *PBS2* (35), which constitutes the MEK element in the cascade. Deletion of *PBS2* had been reported to determine high sensitivity to polymyxin B, and the cells did not survive under conditions of high external osmolarity, showing growth arrest and aberrant morphology (34). Additional data point to an antagonism between *PBS2* and *RAS2* that would involve this gene in the nutrient availability response pathway controlled by *RAS* genes (34). The *SLT2/MPK1* cascade, on the other hand, has been reported not to depend on Ras, at least for its role in modulating the cell cycle arrest response to a lack of nutrients (99).

Knowledge of a system that could be the sensor, or one of the sensors, for this pathway controlling the activity of the cascade has advanced substantially with the discovery of genes *SLN1* and *SSK1* (281). The relationship with the osmosensing pathway became apparent when two suppressors of *sln1* mutants were identified as the *HOG1* and *PBS2* genes. Another suppressor of *sln1* mutations was identified as *SSK1*, which seems to encode the companion response regulator of the Sln1p histidin kinase. The products of *SLN1* and *SSK1* are quite similar to bacterial sensing elements known as two-components systems. A serine/threonine phosphatase, the product of the *PTC1* gene (280), also seems to operate in this pathway (214), probably by dephosphorylation of the MAP kinase, leading to inactivation or attenuation of the signal.

The most outstanding fact with regard to the relationship between the cell integrity/proliferation and the osmosensing MAP kinase pathways has been found in the course of studies that monitored the osmotic stress responses. Gustin et al. (172) have found that high-osmolarity conditions not only activate the osmosensing pathway but also prevent tyrosine phosphorylation of Slt2p. However, the opposite takes place under hypotonic shock, which leads to an accumulation of tyrosine phosphorylation of this MAP kinase, and the phenomenon is dependent on the functionality of the cell integrity/proliferation pathway. It follows that the two pathways we are considering, cell integrity/proliferation and osmosensing, would be regulated in response to osmotic alterations in the external medium. The former would respond to hypotonic conditions, while the latter is activated by hypertonic shocks (172).

Connecting the *PKC1* cascade to cell cycle and morphogenetic functions. The work of Mazzoni et al. (293) points toward the importance of the *SLT2/MPK1* function in polarized growth. These authors have observed that *slt2* mutant strains growing at a nonpermissive temperature display a large number of abnormally small or large cells, an alteration in the pattern of chitin deposition, an abnormal distribution of actin, and an accumulation of secretory vesicles, a phenotype which resembles that of *pfy1*, *cap1*, *cap2*, *sac6*, *bem1*, *rho3*, and *rho4* and, in general, mutations in genes essential for polarized growth (see above). All these facts suggest that Slt2p might play a role in the correct cytoskeletal function for polarized growth, which is consistent with the observation of the lack of viability of *slt2 act1* or *slt2 myo2* double mutations (293). The same authors isolated the mutant allele *slt2-1* as an enhancer of the cell division defect of *cdc28-109* mutants (293), consistent with the previously reported need for Cdc28 activation for the initiation of budding (259). This observation would suggest that the *SLT2* pathway is regulated by protein kinase Cdc28 or, alternatively, that both *SLT2* and *CDC28* act in parallel in the promotion of the budding process (293). The same authors reported the evidence that the defective phenotype of a *cdc28-4* mutant, in which the specific G_1 functions are affected, is enhanced by an *SLT2* deletion, but this does not occur in *cdc28-1N*, which is specifically defective in G_2/M functions. This fact would place the action of *SLT2* in the G_1 phase (293). Furthermore, Lee et al. isolated a gene named *BCK2* in a screen for multicopy suppressors of the *slt2/mpk1* trait. Overexpression of this gene also suppresses a *pkc1* mutation (251). Interestingly, Bck2p is a modulator of the expression of the G_1 cyclins (116). The genetic interaction between *SLT2/MPK1* and *BCK2* implies two possibilities: (i) the existence of two parallel pathways with common transcriptional targets and (ii) the participation of the PKC-led pathway in the transcriptional events regulated by Bck2p. A complete clarification of these appealing results must await the molecular characterization of the phosphorylation target(s) of Slt2p.

Protein phosphatases involved in morphogenesis. Although more than 50 protein kinases have been characterized in *S. cerevisiae*, only a few protein phosphatases are known. Genes coding for protein phosphorylating/dephosphorylating enzymes with a potential role in yeast morphogenesis are listed in Table 6. Mutant phenotypes and genetic interactions suggest that type 2A protein phosphatase activity (PP2A), which has been reported to regulate glycogen metabolism (91), is involved in morphogenetic control as well. *PPH21* and *PPH22* are two PP2A catalytic subunit-encoding genes that, together with the *PPH3* gene, were shown to be important for cell growth and morphogenesis (373). The lack of any one of these genes caused no noticeable phenotype, but when both *PPH21* and *PPH22* were deleted, cell growth was impaired, and the triple mutant *pph21 pph22 pph3* was not viable. When overexpressed, these genes caused dose-dependent changes in cell shape (373), leading to huge, round, multinucleate cells (a *bem2*-like phenotype). Recent studies of a *pph21* temperature-sensitive allele show that actin and chitin are mislocalized at nonpermissive temperatures, revealing a role for PP2A in actin cytoskeleton organization. Putative A and B regulatory subunits for PP2A lie in *TPD3* and *CDC55*, respectively (187, 434). Both genes, when disrupted, show a cold-sensitive aberrant phenotype, with multibudded and multinucleated cells, positively suggesting a role for PP2A in morphogenesis. A *cdc55*-like phenotype is exhibited by a thermosensitive *yck1 yck2* double mutant, and *yck* mutations are lethal in the background of *cdc55* mutations (364). *YCK* genes encode casein kinases. PP2A and casein kinases could account for dephosphorylation/phosphorylation reactions on common substrates for the regulation of cell shape. *CDC55* is thought to participate in the regulation of cytokinesis, but, remarkably, a *cdc55* mutant can be suppressed by mutant alleles of the Rho-GAP *bem2* (see above) (187). In addition to these data, a mutation in *GRR1*, a gene involved in the control of glucose uptake and turnover of G_1 cyclins (20, 147), is synthetic lethal with *cdc55* and is able to partially suppress a *bem2* phenotype (229). Thus, it is likely that the Bem2p function is regulated either by direct or indirect phosphorylation.

SRK1/SSD1 is a gene coding for a protein related to phosphatase function which was isolated as a suppressor of the defects caused by deletion of *SIT4*, a PP2A-like phosphatase gene (415). Sit4p has been thought to operate on cell cycle

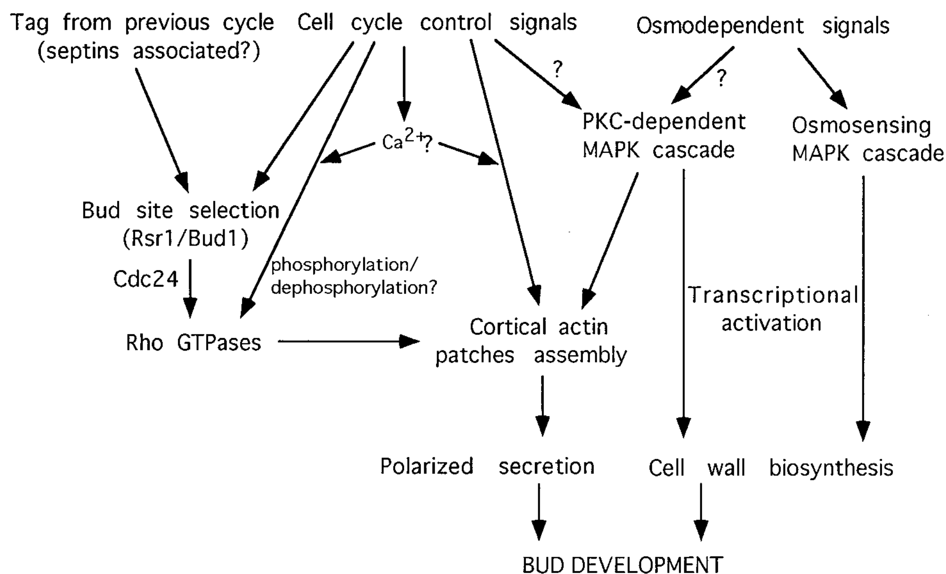


FIG. 8. A potential sequence of the different processes that control morphogenesis for the initiation of budding. Question marks are placed in the most unsettled links. This model presents the dependence of bud emergence on both the process of polarized secretion and the activation of cell wall biosynthetic machinery. How the two processes are correlated is not known. It is expected that the triggering of both processes depends on common signals elicited by cell cycle controls. See the section on a global view in the text for further information.

regulation (116), but a morphogenetic role for this protein has been suggested on the basis of the lethal phenotype of *bem2 sit4* double mutants (229). A relationship of *SRK1/SSD1* with the *PKC1* cascade is suggested by the facts that its deletion in an *slt2* background enhances the impairment of growth and that allele *SSD1-v1* partially suppresses lysis in a *slt2-1 ssd1-d* background (252). Furthermore, a similar phenomenon occurs in a *bem2 ssd1-d* background (229).

Recently, a new aspect of morphogenesis in *S. cerevisiae* has been unveiled: its ability, under certain conditions, to develop pseudohyphal growth (166). This phenomenon occurs only under certain starvation conditions and results in the development of chains of elongated cells that extend into the surrounding medium. Liu et al. (271) have reported that some of the genes involved in the response to mating pheromone in haploids also work in diploids in any function related to this dimorphism (Fig. 7). Some recent experiments suggest that Cdc55 and therefore PP2A activity could be of relevance for this dimorphic transition too. This assertion is based on the observation that *cdc55* mutations are synergic with those in the *ELM1* gene, which was isolated in a screen for mutations that constitutively expressed a filamentous growth (31). Furthermore, overexpression of *PAM1*, a gene that suppresses the growth defect in PP2A-deficient strains, causes a filamentous growth pattern reminiscent of pseudohyphae (201). In spite of these results, we are still ignorant of the precise role played by PP2A activity in cell surface growth regulation.

A type C protein phosphatase activity (PP2C) is represented in yeast cells by the *TPD1* gene product (365). Strains deleted for this gene show multibudded cells, thus displaying a failure in cell separation. The controls of cell separation are poorly understood, and no conclusions can be reached from the present data. By contrast, another PP2C-like serine/threonine protein phosphatase, Ptc1p (280), has been reported to work as a potential modulator of the signal transduced by the osmosensing MAP kinase cascade (214) (Fig. 7).

Other putative protein phosphatase elements manifest a genetic relationship with the *PKC1* pathway. *PPZ1* (348) and *PPZ2* (103) are two yeast phosphatase genes which were identified independently. Deletion of both genes leads to an autolytic caffeine-sensitive phenotype, which is rescued by the presence of an osmotic stabilizer (348) and is similar to phenotypes of strains deleted in genes of the *PKC1* cascade (204). Moreover, the *PPZ2* gene has been cloned as a multicopy suppressor of *slt2/mpk1* deletion strains and has been shown to suppress lysis associated with thermosensitive alleles of *bck1/slk1* and *pkc1* (251). The relationship of these two phosphatase genes with the *PKC1* pathway seems clear, although it is not possible, with the evidence available, to locate their action at a specific place(s) in the phosphorylation cascade. It has been proposed that the Ppz protein phosphatases could act on a pathway parallel to the one led by Pkc1p (251).

GLOBAL VIEW: CHASING THE LINKS

A vast array of yeast genes with a function presumably related to morphogenesis has been revealed. As deduced from biochemical studies, some of them have an obvious implication in cell wall biosynthesis, actin polymerization, or signal transduction. However, in most cases, this implication is based on only genetic evidence or in vitro studies, but the particular in vivo function of many of the genes isolated so far remains obscure. The challenge now is to fill in the gaps and relate cell signalling to the polarization of growth and to wall biosynthesis.

Figure 8 ventures a glance at the global sequence that leads to cell surface growth during budding. In this model, we propose that bud emergence should be promoted by two simultaneous events: polarized secretion and the onset of cell wall biosynthesis. How both processes are coordinated in the cell is not currently understood, although important hints have recently emerged (see below). Polarized secretion is conducted by the cortical cytoskeleton, a complex system of actin filaments and actin-associated proteins whose assembly is controlled by Rho proteins. The function of Rho GTPases may respond, or run parallel, to signalling pathways in which phos-

pholipids and free calcium are involved. Remarkably, interactions of Rho proteins with unconventional myosin (358) and protein kinases involved in signal transduction (288) have been recently found in mammalian cells. The undermembrane spot in which all these events must take place is probably marked by specific remnants from the last cell cycle, and temporal control is achieved by START-dependent signals. The PKC-led and osmosensing MAP kinase cascades may be counterbalancing the regulation of the same events in response to the status of cell surface.

One of the common links between β-glucan synthesis activation and polarized secretion could be a potential regulation by the calcium levels (see the section on a role for calcium, above), but other possible links are coming into sight. Remarkably, the latest findings extend the importance of small GTP-binding proteins to the regulation of 1,3-β-glucan synthesis. Particularly, the outstanding results of Mol et al. provide biochemical evidence for the implication of a small GTP-binding protein in the direct regulation of 1,3-β-glucan synthase (300). In addition, a functional and structural homolog of the Rho GGTase encoded by *CDC43*, which complements the defects of a fission yeast lytic mutant showing a low level of β-glucan synthase activity, has been cloned in *S. pombe* (114, 359). In addition, the products of the genes *MSB2*—a multicopy suppressor of *cdc24*—and *HKR*—encoding a putative modulator of glucan synthesis—show structural similarities that might denote a common function. Moreover, mutants with mutations in *BEM2*, encoding a Rho-GAP, display a weak cell wall (87), which may reflect alterations in its composition. It is worth mentioning in this context that strains bearing mutations in some of the *RHO* genes display a cell lysis defect that can be prevented by osmotic stabilization, thus implying an altered cell wall (291). Anyway, it is still unclear whether the alterations in cell wall and impairment of 1,3-β-glucan synthase activity are an indirect effect of the polarization defect or whether Rho proteins are indeed one of the links that coordinate polarized secretion and cell wall biogenesis.

Spa2p is certainly an intriguing piece in the cellular machinery for cell polarity. It resembles no other sequence and seems nonessential for morphogenesis, except for the selection of polarity and for mating efficiency (162). Nevertheless, it becomes essential in strains lacking diverse genes that share the only feature of being important for morphogenesis, such as septin (*CDC10*), a transcription factor important for START (*SWI4*), protein kinases from the cell integrity/proliferation cascade (*BCK1* and *SLT2*), or bud emergence genes (*BEM1* and *BEM2*) (279). An exciting result is the recent isolation of *MKK1* as a two-hybrid interactor with *SPA2* (100). The possibility that Mkk1p and Spa2p really interact in vivo generates a highly interesting link between cell polarity and the protein kinase C cascade.

Another interesting piece is Gas1p/Ggp1p (347, 352). This GPI-anchored mannoprotein is cell cycle regulated, and its apparently relevant implication in morphogenesis is surprising for a protein which is supposed to be located in the extracellular compartment. It is predictable that this protein plays a key role in the control of cell wall assembly during growth, although we currently ignore its function or its molecular interactions.

Some more encouraging results come from the implication of the osmosensing MAP kinase pathway in β-glucan degradation. Among genes which cause a killer toxin resistance phenotype when overexpressed, *EXG1*, *PBS2*, and *PTC1* were found (214). As stated above, *PBS2* and *PTC1* encode, respectively, a MEK and a phosphatase that mediate in the osmosensing pathway. Interestingly, both *EXG1* mRNA levels and exo-β-glucanase activity are higher when these genes are overexpressed (214), so that killer resistance is probably due to a higher Exg I activity in all three cases. Other studies point out that Bgl2p, encoding another β-glucanase (see above), is partially responsible for the morphogenetic defects of protein kinase C mutants, since a *bgl2* mutation partially suppresses a *pkc1* growth defect (397). The biological significance of these results awaits the elucidation of the precise role of glucanases in cell wall dynamics.

Further results that link the cell integrity/proliferation signalling cascade to cell wall synthesis are the genetic interactions between the genes involved in 1,6-β-glucan synthetic pathways and those coding for protein kinases of this cascade (369) (Fig. 6). *pkc1* defects are rescued by *SKN7* (47) and *KRE6* (369), and synthetic lethality is observed in strains with double deletions in *pkc1* and *kre6*, *pkc1* and *skn1*, *pkc1* and *kre11*, *pkc1* and *kre2*, or *slt2* and *kre6*, and triple *mkk1 mkk2 kre6* or *mkk1 mkk2 kre11* mutants (369).

Another point of interest is the recently unveiled ultrastructure of the actin filaments-plasma membrane association (306), which will probably contribute to our understanding of the last stages of polarized secretion. A possible role of the reported finger-like invaginations at the sites of actin assembly would be to reduce the local turgor pressure at the growing spots (306), where the developing cell wall should be less protective, as has been said to occur in some bacteria (232). Whether direct or indirect, a link between cell wall structure and the actin cytoskeleton is given by the work of Gabriel and Kopecká, who showed that the texture of the glucan matrix is abnormal when an actin mutant phenotype is expressed (156a, 156b), suggesting that the actin-plasma membrane complex is indeed important for a proper assembly of the cell wall polymers.

One of the most fascinating aspects of yeast morphogenesis is the accurate disposition of chitin at the mother-daughter junction and in the primary septum (Fig. 2A and B, panel 1). As stated above, CSIII is responsible for the bulk of chitin synthesis, including the ring, and spatial as well as other evidence points to the possibility that septins are involved in the control of chitin deposition (see above). In any case, since mutations that specifically affect the assembly of the actin cytoskeleton also provoke an aberrant deposition of chitin, it is likely that both septins and actin are involved in the polarization of chitin synthesis. CSII seems responsible for chitin deposition at the septum (see above), and it is interesting that both Act2p, which may have a specific role in the control of septation, and Chs2p (CSII) bear putative phosphorylation targets for the Cdc28p kinase. Septin mutants are defective in the localization of the septum, meaning that the neck microfilaments may also mediate in this process. The control of cell separation may depend on the regulation of chitinase, whose hydrolytic function is counterbalanced by CSI (see above).

Morphogenesis in the shmoo is also starting to be revealed (see reference 78 for a review), and a connection of the mating pheromone response pathway to the polarization of growth for shmoo formation may be given by the recent isolation of a novel putative GAP for Cdc42p, encoded by *RGA1*, whose loss constitutively activates the mating pheromone-dependent phosphorylation cascade (408a). An interesting observation is that the human homologs of Cdc42p and Ste20p physically interact (288). If this is demonstrated to occur in yeast as well, we would be able to connect cell surface morphogenetic signalling to pathways that regulate transcriptional events. Other complex morphogenetic processes, such as sporulation and pseudohyphal growth, also deserve our attention. Nevertheless, the amount of data presented so far is very limited. Sporulation is known to involve specific cell wall biosynthetic and

hydrolytic structural or regulatory components, like Ssg1p or Sch1p (see above), but the control of spore morphogenesis is almost an untouched field. Some light was shed on this subject when two novel protein kinases, a Ste20-homolog (Sps1p) and a MAP kinase (Smk1), related to sporulation were recently found (155, 276). Strains deleted in *SPS1* show a spore wall lacking the chitosan and dityrosin-rich layers (155), whereas deletion of *SMK1* causes an aberrant and clearly unprotective spore wall in which the layered structure is still manifest but the disposition of the layers is inappropriate (276). A very appealing idea is that specialized MAP kinase cascades would be controlling distinct aspects of morphogenesis through the yeast life cycle (Slt2p and Hog1p for budding, Fus3p and Kss1p for mating, Smk1 for sporulation, and some other yet unknown, although sharing upstream components with the mating pathway, for pseudohyphal growth). Regarding the dimorphic transition for pseudohyphal growth, it sets new challenges for the study of morphogenesis, since cells elongate, cell separation is bypassed, and growth is unipolar, in contrast to the usual bipolar pattern (166, 242).

FINAL COMMENTS AND OUTLOOK

To summarize, morphogenesis in unicellular eukaryotes such as *S. cerevisiae* is a complex process involving many pathways and components. The efforts of many research teams have contributed much to our understanding of many components and events involved in morphogenesis and cell wall deposition, but many enigmas remain. For example, we do not understand how cell wall assembly is coordinated during growth, how Rho proteins function to regulate the actin cytoskeleton, or how the recently unveiled phosphorylation pathways obey environmental or internal signals, eventually regulating cell wall biosynthetic events for the maintenance of an osmotic balance. Further genetic and biochemical characterization of new components and a deeper knowledge of the functions of the current ones and the interactions among them will undoubtedly help to elucidate the mechanisms by which these events occur.

The analysis of cell wall assembly and its regulation can be of great relevance to the development of research in antifungal agents of clinical interest, and the study of yeast cell lysis may contribute to the solution of some current problems with certain biotechnological processes. In addition, understanding how morphogenesis is achieved in simple cells will provide us with important clues to interpret how higher cellular systems adapt their shape to environmental challenges.

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