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MAP Kinase Pathways in the Yeast *Saccharomyces cerevisiae*

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INTRODUCTION

Despite their placid appearance, cells of the yeast *Saccharomyces cerevisiae* possess rapidly responding, highly complex signaling pathways. These pathways allow yeast cells to quickly adapt to a changing environment, a critical attribute for a nonmotile species. Prominent among yeast signaling pathways are the mitogen-activated protein kinase cascades (169, 249). These generally contain three protein kinases that act in series: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MEK), and a MAP kinase (MAPK) (66, 71, 290). Thus, when the cascade is activated, the MEKK phosphorylates the MEK, which in turn phosphorylates the MAPK. The MAPK cascades, found in animals (71, 290), plants (173), and fungi (118, 169), often regulate transcription factors by MAPK-mediated phosphorylation. Many extracellular and intracellular signals modulate transcription of specific genes through activation or inhibition of MAPK cascades.

Our understanding of the *S. cerevisiae* MAPK pathways is

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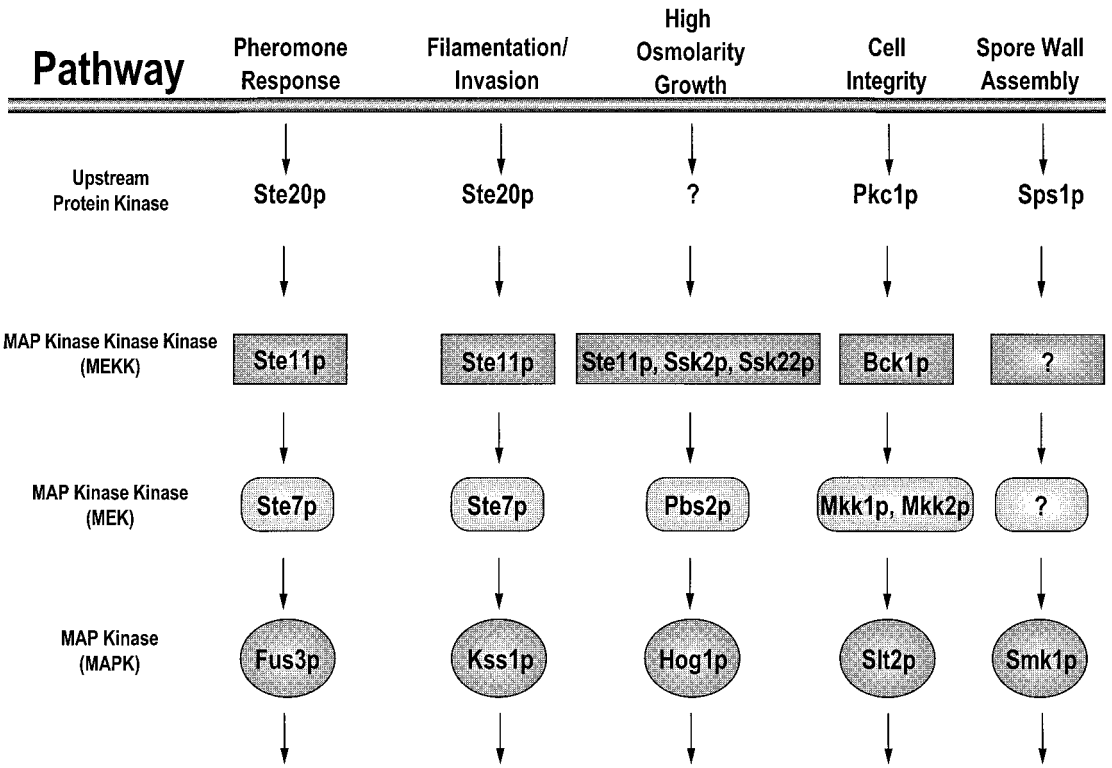

FIG. 1. MAPK cascades of *S. cerevisiae*. There are four MAPK pathways in vegetatively growing yeast and one, the spore wall assembly pathway, which is expressed only in sporulating yeast. Nomenclature for yeast genes and their products is as follows: *STE20*, gene name; *ste20*, recessive mutation; *ste20*Δ, deletion (usually null) mutation; and Ste20p, protein product of *STE20*. The question marks indicate that a protein kinase has not yet been identified for this step in a cascade. Note that each cascade has a unique MAPK. In addition, certain protein kinases act in more than one pathway: the MEK Ste7p (two pathways), the MEKK Ste11p (three pathways), and the upstream MAPK cascade activator kinase Ste20p (two pathways). The arrows represent known or postulated steps in signal transduction; see the text for details.

more complete than that of MAPK pathways in other organisms. Extensive genetic and biochemical analysis plus the complete sequencing of its genome has revealed that *S. cerevisiae* contains five MAPKs on five functionally distinct cascades (Fig. 1) (179). Four of these pathways, the mating pathway, the filamentation-invasion pathway, the cell integrity pathway, and the high-osmolarity growth pathway, are present in growing cells. The Smk1p MAPK, part of the spore wall assembly pathway, is not present in growing cells but appears during sporulation and regulates that developmental process. Another type of yeast, the fission yeast *Schizosaccharomyces pombe*, contains a set of MAPK cascades that have some similarity to those in *S. cerevisiae*. Although this review is focused on *S. cerevisiae* MAPK pathways, some similarities and, more importantly, differences between two related MAPK pathways in these two evolutionarily diverged yeasts are discussed. In this review, *S. cerevisiae* cells will be called yeast or budding yeast and *S. pombe* cells will be called fission yeast.

The biochemical mechanisms mediating signal transduction among the three types of kinases in MAPK cascades are fairly well understood (397). MEKK has a regulatory domain at the NH_2 terminus and a protein kinase domain at the COOH terminus. When activated, MEKK phosphorylates both a serine and a threonine residue in a conserved domain in the NH_2-terminal portion of MEK. The phosphorylated and now activated MEK then phosphorylates MAPK on a threonine and a tyrosine residue, separated by a single amino acid, within the activation loop (199) of the conserved kinase domain, thereby activating the kinase activity.

Different classes of MAPKs exist in yeast and also in mammals. These can be classified by the pathways in which they participate and by the identity of the amino acid between the Thr and Tyr in the activation loop: Glu, Pro, or Gly in mammals, and Glu, Gly, or Asn in yeast. For example, the ThrGlyTyr MAPKs such as yeast Hog1p or mammalian p38 are found in stress-activated pathways and the ThrGluTyr MAPKs such as yeast Fus3p or mammalian ERK1 are found in growth factor-activated pathways (230, 397). Although the amino acid between the Thr and Tyr can be used to classify different MAPKs, other regions of the conserved protein kinase domain appear to play a more dominant role in determining the specificity of interactions with the upstream MEK and downstream substrates (49).

Despite a wealth of information on the MAPK cascade itself, there are many unsolved problems concerning this signaling device. The way in which the known upstream activators act on the cascade is still unclear. Identification of new target proteins for the MAPKs and novel activators of the MAPK pathways is still continuing. MAPK cascades appear to exist in cytoplasmic macromolecular complexes with other proteins that serve as scaffolds, anchors, or adaptors. Upon activation, MAPK or MEK is thought to move from the cytosol to the nucleus and phosphorylate target proteins such as transcription factors. How the cytoplasmic complexes of signaling proteins rearrange themselves during signaling to let MAPK or MEK go to the nucleus is not well understood. It is still unclear what determines the speed, magnitude, specificity, and duration of signaling through a MAPK cascade. The mechanisms by which signaling through MAPK pathways is integrated with that through other types of pathways is just starting to be

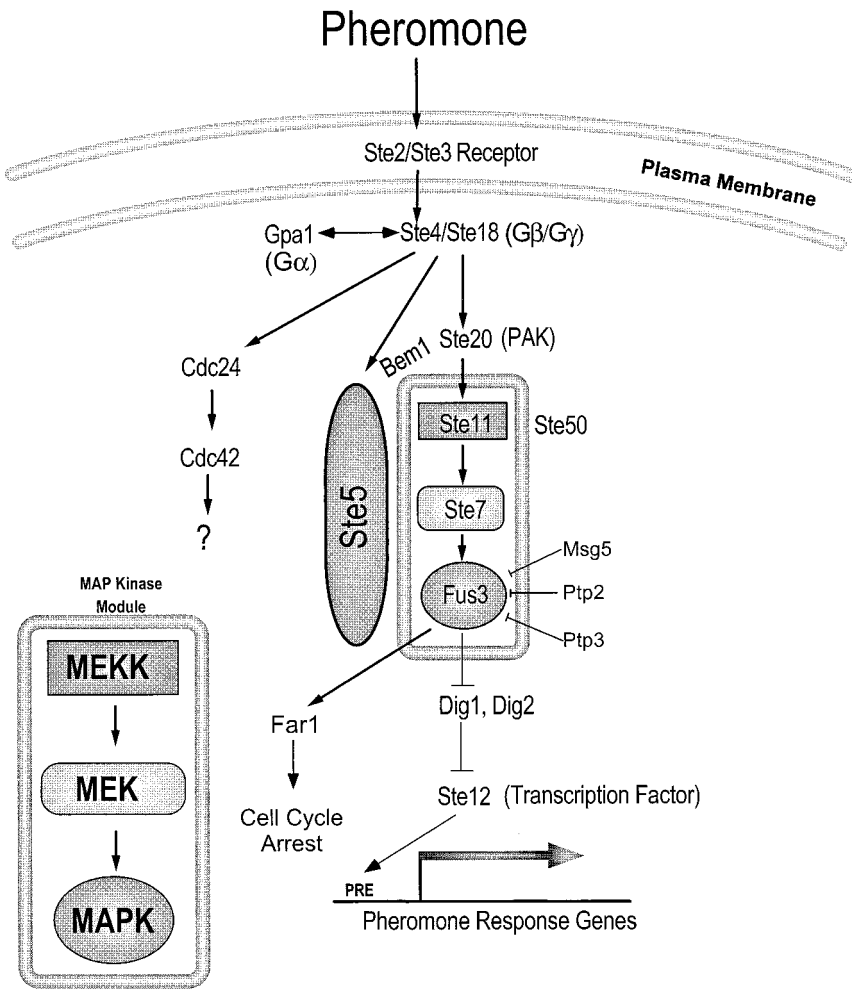

FIG. 2. Pheromone response pathway of *S. cerevisiae*. The line with arrows connecting Gα to the GβGγ indicates the ability of the protein subunits to form a complex in the absence of pheromone. →, activation; ⊣, inhibition (these connections do not necessarily mean direct physical interactions). Proteins are labeled without the p suffix (e.g., Ste5 instead of Ste5p) to improve the legibility of the figure. See the text for details of signal transduction between different proteins on the pathway.

studied. The yeast MAPK pathways are better characterized than those in other eukaryotes. The general principles of operation and the variations of this simple signaling cascade revealed in yeast and described here may thus help guide research on similar pathways in other eukaryotes. Each of the five yeast MAPK-containing pathways is discussed, starting with the mating-pheromone response pathway, the best understood of all eukaryotic MAPK pathways.

MATING-PHEROMONE RESPONSE PATHWAY

Yeast cells can exist as either haploid or diploid cells. Haploid cells of the opposite mating type (**a** or α) can mate, i.e., fuse and form a diploid. This process is stimulated by the release of small peptide mating pheromones, **a**-factor from *MAT***a** cells and α-factor from *MAT*α cells, that act on cells of the opposite mating type to prepare that cell for mating. Cellular responses to mating pheromone include polarized growth toward a mating partner, cell cycle arrest in G_1, and increased expression of proteins needed for cell adhesion, cell fusion, and nuclear fusion. A pheromone-activated signaling pathway that includes a MAPK cascade (Fig. 2) helps mediate many of these responses. Pheromone binds to and activates a seven-transmembrane domain receptor that in turn is thought to induce the dissociation of a heterotrimeric G protein (32, 99, 194, 316). As described below, the liberated Gβ(Ste4p)-Gγ(Ste18p) complex then activates downstream proteins Ste5p and Ste20p, and these in turn stimulate the Ste11p-Ste7p-Fus3p MAPK cascade. The MAPK Fus3p phosphorylates several downstream targets, e.g., Far1p, Dig1p, Dig2p, and Ste12p, that mediate various responses required for successful mating.

Activation of the MAPK Cascade

Gβ activation of the mating-pheromone MAPK cascade is mediated primarily by Ste5p (51, 235, 446, 487) and Ste20p (236, 237, 499). Other proteins, such as Ste50p (387, 502) and Bem1p (204, 234, 243, 270), may also play a role in transducing signals from the Gβ Ste4p, but their functions are not essential. Ste5p and Ste20p appear to be necessary and limiting for MAPK cascade activation by Gβ (3, 61, 164, 218, 235). The way in which these proteins cooperate is not yet understood, but for each mediator there is some information about the transduction mechanism.

Ste5p, a scaffold for the MAPK cascade. Organization of signal transduction along pathways commonly involves scaffold, anchoring, or adapting proteins (364). Several lines of

evidence argue that Ste5p is a scaffold protein for the pheromone-activated MAPK cascade. Ste5p associates with MEKK Ste11p, MEK Ste7p, and MAPK Fus3p (also MAPK Kss1p) in the two-hybrid system (61, 187, 287, 383) and in coprecipitation experiments (61, 218). Although these interactions could occur independently or indirectly, two observations suggest that Ste5p is a scaffold. First, Ste5p has separate binding sites for the different protein kinases (61, 187), and second, Ste5p appears to exist in a high-molecular-weight complex with these kinases (61), which has a high-specific-activity Fus3p kinase (62, 218). The sites of interaction of Ste5p with different protein kinases have been identified. Analysis of point mutations and deletions that block specific interactions (61, 187) showed that a single Ste5p polypeptide of 917 residues has separate sites required for binding Ste11p (residues 463 to 514), Ste7p (residues 744 to 895), and Fus3p (or Kss1p) (residues 241 to 336), respectively. Importantly, a mutation in a Ste11p binding site blocks signal transduction in the pathway, as revealed by a failure to complement the sterile phenotype of a *ste5*Δ strain, supporting a positive regulatory role for the scaffolding function of Ste5p in signaling (187).

A scaffold protein such as Ste5p could increase the specificity of the kinase cascade by blocking inappropriate interactions with other, related kinase cascades. For example, the MEKK Ste11p also acts on a different MAPK cascade, the HOG pathway (378). Pheromone-activated Ste11p is unlikely to diffuse freely in the cell, because Ste11p forms very stable complexes with Ste5p (61). If Ste11p were free to move, pheromone might activate the HOG pathway. By bringing together Ste11p and Ste7p and facilitating Gβ activation of Ste11p (see below), the Ste5p scaffold may enslave part of the cellular Ste11p to a specific role in the pheromone response pathway. This possibility is supported by observations suggesting that Ste5p may restrict the function of Ste7p. A constitutively active MEK Ste7p will activate another MAPK on a separate pathway only when the mutant protein is overexpressed or present in low copy when Ste5p is missing (510).

The specificity of interactions between different components of the MAPK cascade is not solely determined by their association with Ste5p. For example, the MEK Ste7p interacts with Fus3p and the related MAPK Kss1p independently of Ste5p (19, 61, 383). The Ste7p-Fus3p (or Kss1p) interaction is quite specific because two other *S. cerevisiae* MAPKs, Hog1p and Slt2p, do not interact with Ste7p (19). The interaction between Ste7p and Fus3p (or Kss1p) could be an enzyme-substrate interaction involving binding of the MAPK to the catalytic site of the MEK. However, because Ste7p is a substrate of Fus3p (and Kss1p) as part of a potential feedback mechanism (see below) (19, 119), this interaction could also reflect the binding of the MEK to the catalytic site of the MAPK. Neither of these possibilities accounts for the strong interaction between MEK and MAPK. Instead, Fus3p (or Kss1p) binds tightly to an NH_2-terminal region of Ste7p that contains no phosphorylation sites and is not part of the COOH-terminal kinase domain (19, 21).

How the MEKK Ste11p is activated is an important question for the pheromone response pathway. It is striking that the Ste11p kinase (MEKK) is as active in vitro when isolated from either control or pheromone-treated cells, as assayed by phosphorylation and activation of the Ste7p kinase (MEK) (334). This finding suggests that Ste11p is regulated in vivo by a negative regulatory factor(s). Ste11p has two domains: a COOH-terminal protein kinase domain and an NH_2-terminal regulatory region. Genetic evidence argues that Ste11p activity is negatively regulated by its NH_2-terminal regulatory domain. Deletion mutations or a specific point mutation in the regulatory domain induces constitutive activation of the mating-pheromone pathway (51, 446). Ste5p interacts with the NH_2-terminal negative regulatory domain of Ste11p in the two-hybrid system (61, 383) and is therefore in a position to counteract this negative regulation. Consistent with this possibility is the observation that an activating mutation in the NH_2-terminal domain of Ste11p strongly increases the interaction between Ste11p and Ste5p (383). Thus, the role of Ste5p may be not just to facilitate interactions between protein kinases of the MAPK cascade but also to directly regulate kinase activity.

(i) Oligomerization of Ste5p. Several studies have shown that Ste5p forms homo-oligomers in yeast. Because protein oligomerization has been implicated as a signal transduction mechanism in several systems (245, 322), this facet of Ste5p has received some attention. The existence of Ste5p oligomers was first suggested by observations of interallelic complementation of different *ste5* mutants that did not complement a *ste5* deletion on their own (505). The results of two-hybrid analysis and coprecipitation experiments confirmed the existence of Ste5p oligomers (127, 188, 505). Oligomerization of Ste5p does not require the MAPK cascade (505) and appears to be independent of mating pheromone (127, 505). Two domains of Ste5p, both located in the NH_2 terminus of the protein, mediate oligomerization (505). One domain (residues 335 to 586) overlaps the Ste11p-binding region, and the other (residues 139 to 239) contains a LIM (91, 404) or RING-H2 (40) domain. The LIM domain appears not to be essential for oligomerization, because *ste5* mutants harboring deletions of the LIM domain still oligomerize efficiently based on two-hybrid analysis (505) and coprecipitation experiments (127). Two-hybrid analysis suggests that residues NH_2-terminal to the LIM domain may be essential for oligomerization (127, 283, 505).

Mutations that have been demonstrated to solely block Ste5p oligomerization have yet to be described; therefore, the role that oligomerization plays in signal transduction is not yet clear. Nevertheless, several results argue that oligomerization may be required for signal transmission though the MAPK cascade. Different fragments of Ste5p that are predicted to be defective in binding to one or more kinase show interallelic complementation (505). More recent work shows that coexpression of two different nonfunctional Ste5p point mutants, one that cannot bind Ste7p and one that cannot bind Ste11p, fully complements the sterile phenotype of *ste5*Δ (187), providing strong evidence that oligomerization is important for signal relay from Ste11p to Ste7p.

The ability of *ste5* mutants defective in different kinase binding sites to restore Ste5p function predicts that the mating-pathway MAPK cascade functions quite well if the MEKK bound to one Ste5p polypeptide is allowed to phosphorylate only an MEK bound to another Ste5p polypeptide. Whether this is the normal mechanism of MEKK-MEK interaction on the pheromone response pathway is unknown. Growth factor-activated tyrosine kinase receptors (245, 470) are activated by a homo-oligomerization-dependent mechanism in which the protein kinase domain on one receptor polypeptide chain phosphorylates not itself but a site on another, identical polypeptide. Perhaps the Ste5p-MAPK cascade works in a similar fashion. Ste5p-bound Ste11p may be sterically hindered from phosphorylating Ste7p bound to the same Ste5p polypeptide, and dimerization is required to bring together kinase and substrate.

(ii) Gβ activation of Ste5p. Gβ appears to activate the MAPK cascade through a direct interaction with Ste5p. Pheromone stimulates the binding of Ste4p to Ste5p (127), with Ste4p binding at the NH_2 terminus of Ste5p (487). Mutations in conserved cysteine residues of the RING-H2 domain block

Ste4p binding (127, 188). These mutants are sterile and block pheromone-induced signal transduction (127, 188), although they still efficiently interact with Ste11p, Ste7p, and Fus3p (127, 188).

The activation of Ste5p and its associated MAPK cascade by the Gβ Ste4p may be related to the oligomeric state of Ste5p. The NH_2-terminal LIM domain of Ste5p that appears to bind Ste4p (127, 188, 487) overlaps a part of Ste5p that is required for oligomerization (127, 188, 505). LIM domain point mutations either inhibit (188) or stimulate (127) Ste5p oligomerization, depending on whether two cysteines or one cysteine is mutated to alanine, respectively. This coincidence of sites suggests that Ste4p may regulate Ste5p oligomerization. Fusion of an oligomerization-defective and sterile Ste5p RING-H2 mutant to glutathione *S*-transferase, a protein predicted to dimerize, restores mating to both *ste5*Δ and *ste4*Δ *ste5*Δ mutants, suggesting that Ste5p dimerization is sufficient for activation of Ste5p and the MAPK module (188). Restoration of mating by this fusion protein is much stronger in the *ste4*Δ *ste5*Δ strain, suggesting that Ste4p plays a negative regulatory role (188). Because fusion of glutathione *S*-transferase to Ste5p enhances the basal but not the pheromone-induced activity of the MAPK cascade for both *STE5* and a *ste5* LIM domain point mutant (127), oligomerization may play a role in signaling from Ste11p to the MAPK. Still unknown is whether the degree of oligomerization of Ste5p plays a role in binding to Gβ or whether it is regulated by Ste4p in response to mating pheromone (505).

Ste20p regulation of the MAPK cascade. Upstream protein kinases that activate MAPK cascades have been identified in the pheromone response pathway, the filamentation-invasion pathway, and the cell integrity pathway. Ste20p is believed to be the upstream kinase that activates the MEKK Ste11p in the pheromone response pathway (236, 390). Ste20p also functions upstream of Ste11p in the filamentation-invasion pathway (257, 395). It is striking that Ste20p appears to have additional functions that are independent of MAPK cascade activation. These Ste20p functions include activation of myosin I function (238, 497, 498), adhesion of mating partners (239), and vegetative functions relating to budding (83) and cell elongation (396). Whether the separate functions of Ste20p are mediated by a single macromolecular complex or by separate protein complexes, each with a uniquely regulated Ste20p, remains unclear.

Signal transduction from the Gβ protein Ste4p to the downstream MAPK cascade requires the protein kinase Ste20p in addition to the previously discussed Ste5p (236, 390). Ste20p is the founding member of the p21-activated kinase (PAK) family (125, 255, 285). Strains with Ste20p deleted are not as completely sterile as a *ste4*Δ mutant (3). Yeast has two Ste20p-related protein kinases, Cla4p and Skm1p (83, 293), and it is possible that one of these p21-activated kinases can partially cover for the loss of Ste20p and allow a low level of mating in a *ste20*Δ strain. The function of Skm1p is not yet clear; Cla4p is required for normal progression through the later stages of cell division (30, 83). An overlap in function between Ste20p and Cla4p is suggested by the observation that the *ste20*Δ *cla4*Δ mutation is lethal whereas either single mutant is still viable (83).

It was initially thought that Ste20p activates the MAPK cascade through interactions with Cdc42p (435, 521), an essential member of the Rho subfamily of Ras-related proteins (157). As described below, Cdc42p is involved in the pheromone response pathway but probably functions through proteins other than Ste20p (238). Ste20p has a protein kinase domain near its COOH terminus and a regulatory domain at the NH_2 terminus. As shown in the two-hybrid system and in biochemical assays, this latter region has a binding site for Cdc42p (239, 369, 435, 521). Cla4p has a similar Cdc42p binding site (83). Cells containing Ste20p but with the Cdc42p binding site deleted have near-wild-type levels of mating and pheromone-induced transcriptional responses (239, 369). Instead, the only obvious defect in these cells is a failure to localize Ste20p to its normal locations, a crescent-shaped area of the emerging bud tip and the tip of the shmoo, the mating projection of the cell (239, 369). These are the locations where Cdc42p is localized (527). Cdc42p therefore appears to function to localize Ste20p. Cdc42p stimulated the in vitro activity of the Ste20p kinase in one study (435); however, two more recent studies argue that Cdc42p-GTP has no in vitro effect on Ste20p kinase activity (369, 521).

How, then, is Ste20p activated by Gβ in pheromone-treated cells? Pheromone stimulation induces the association of Ste4p with Ste20p (244). The association of Ste4p with Ste20p involves a short domain at the COOH terminus of Ste20p, outside of its kinase domain (244). Ste20p thus interacts with two small regulatory proteins, Cdc42p at its NH_2 terminus and Ste4p at its COOH terminus. How the binding of Ste4p regulates Ste20p activity has not been determined. Pheromone treatment does induce the phosphorylation of Ste20p; however, the functional significance of this phosphorylation and the identity of the protein kinase that catalyzes this phosphorylation remain unknown (499). Ste20p autophosphorylation does increase its in vitro kinase activity (499), possibly by relieving the negative regulation from the NH_2-terminal domain (390). However, a Ste20p mutant with defective kinase activity still shows pheromone-induced Ste20p phosphorylation in vivo, suggesting that another protein kinase must be involved (499). This is consistent with genetic evidence suggesting a second pheromone-dependent signal from Gβ that involves Ste5p but not Ste20p (3, 127, 270).

It is tantalizing that we know so much about the proteins on this pathway but there are still so many holes in our knowledge about their signaling function. One such example is the still-mysterious Ste20p-to-Ste11p step. The protein kinase Ste20p will phosphorylate the MEKK Ste11p in vitro, but this does not change the kinase activity of Ste11p (499). One protein that might play a role in this step and that is required for pheromone activation of Ste11p is Ste50p (387, 502). Ste50p interacts in the two-hybrid system with Ste11p (502). Constitutively active Ste11p does not interact with Ste50p (502) but interacts more strongly with Ste5p (383). Ste50p shows sequence similarity to the fission yeast protein Ste4p, which has been shown to interact with the Byr2 (22), the fission yeast homologue of *S. cerevisiae* Ste11p. The function of Ste50p in activation of the MAPK pathway, apart from this Ste11p interaction, remains a mystery. Finally, we do not know how the functions of Ste20p, Ste50p, and Ste5p are coordinated to mediate Gβ activation of Ste11p.

Signaling Pathways and the Cytoskeleton

Cellular localization and activation of the pheromone-activated MAPK cascade appears to involve proteins that are functionally connected to the cytoskeleton. This is a common observation in eukaryotic signal transduction. For example, tethering of signal transduction proteins to particular regions of the cell is mediated in part by the cytoskeleton. Also, some signaling pathways regulate the function of the cytoskeleton and, in certain situations, the cytoskeleton participates in transmitting signals to the nucleus. One system in which these different cytoskeleton-signal transduction relationships have

been well explored is the pheromone response pathway in yeast. Two proteins in particular, Cdc42p and Bem1p, connect the pheromone response pathway to the actin cytoskeleton.

Cdc42p is required to orient the actin cytoskeleton to form a bud, to divide the cell during cytokinesis, and to form mating projections (1, 110, 254, 527). Cdc42p therefore interacts with a variety of different proteins that regulate actin cytoskeleton function. Cdc42p in cells exists in a dynamic equilibrium between the GDP-bound and GTP-bound forms. Exchange of GDP for GTP on Cdc42p is activated by Cdc24p (525), and the hydrolysis of the Cdc42p-bound GTP to GDP is predicted to be regulated by the GTPase-activating proteins (GAPs) Bem3p (525) and Rga1p (445). Cdc24p, like Cdc42p, is an essential protein required for polarized cell growth during bud formation and formation of mating projections during conjugation (60, 439).

Several observations suggest that Cdc42p plays an important role in the pheromone response pathway. Temperature-sensitive *cdc24* or *cdc42* mutants, when grown at a nonpermissive temperature, do not show an increase in *FUS1-lacZ* expression (*FUS1* is a pheromone-induced gene [303, 466]) in response to pheromone treatment (435, 521) and cannot mate (393). Strains with the Cdc42-GAP Rga1p deleted show increased pheromone-induced transcription (445). Indeed, overexpression of a mutant Cdc42p locked in the GTP-bound state activates *FUS1* expression (435, 521), even in a strain carrying a dominant negative mutant of the Gβ Ste4p (435). The increased *FUS1-lacZ* expression in cells expressing an activated Cdc42p does require the presence of pheromone, suggesting that Cdc42p acts to modulate signaling by the pheromone response pathway.

Cdc42p appears to have multiple functions in the mating response, at least one of which does not involve the MAPK pathway. Yeast cells form mating projections in response to pheromone treatment. The growth of these projections is spatially oriented toward the source of pheromone and is therefore called chemotropic growth (416). This process involves activation of Cdc42p (335) but, importantly, does not require the protein kinases of the MAPK cascade (410). As discussed above, Gβ Ste4p interacts with Ste5p and Ste20p and, by mechanisms yet unclear, activates the MAPK cascade. Ste4p also interacts with Cdc24p (335, 521), the guanine nucleotide exchange factor for Cdc42p. Mutations in Cdc24p that block the interaction with Ste4p also block chemotropic growth but have no effect on other responses to pheromone including MAPK cascade-mediated growth arrest and *FUS1-lacZ* expression (335). Because the function of Cdc24p is to activate Cdc42p and Cdc42p mediates polarized cell growth, the interaction of Gβ with Cdc24p may provide a mechanism to locally activate Cdc42p and Cdc42p-dependent growth in the vicinity of pheromone-occupied receptors.

Bem1p, like Cdc42p, interacts with several proteins important for the function of the actin cytoskeleton in polarized growth (28, 57, 59, 110). Bem1p associates with actin and with the pheromone response pathway-signaling proteins Ste5p and Ste20p (243, 270). The Bem1p-bound Ste5p is complexed to the Ste11p-Ste7p-Fus3p MAPK cascade (270). Interaction of Ste20p with Bem1p is required for association of Ste20p with actin (243). The fraction of these signaling proteins associated with macromolecular complexes in the cell is considerable. At least half of the cellular Ste5p, Ste20p, and Bem1p localizes to a particulate fraction of the cell and remains there after extraction of membrane proteins with nonionic detergents (243). Bem1p interacts in cells with other signaling proteins: the Cdc42p guanine nucleotide exchange factor Cdc24p (298, 370); Far1p (270), a protein needed for pheromone-induced cell cycle arrest (55) (see below); and Boi1p and Boi2p (29, 298), proteins involved in the regulation of the Rho-type GTPase Rho3p and Rho3p-dependent growth-related processes.

Bem1p-associated proteins can have more than one function. For example, Far1p has two functional parts, a COOH-terminal domain required for chemotropism (107, 473) and an NH_2-terminal domain required for pheromone-induced cell cycle arrest (473). The observation that the MAPK cascade is required for cell cycle arrest (113) but not chemotropism (410) provides further confirmation that these are mechanistically separate responses to pheromone. The mechanism by which Far1p performs two very different functions is unknown. Thus, the multitude of interacting partners for Bem1p and their functional diversity raise the question whether a single Bem1p molecule can complex simultaneously with all potential partners or whether different Bem1p molecules form separate complexes with different protein partners.

So far, it has not been possible to detect an effect of pheromone on the extent of interaction between Bem1p, Ste20p, and Ste5p as assayed by coimmunoprecipitation experiments (243, 270). Thus, Bem1p might just simply tether the signaling pathway to the cytoskeleton. Bem1p does, however, facilitate signaling by the pheromone pathway. Deletion of *BEM1* decreases the pheromone-induced transcription of *FUS1* (204, 270). In addition, overexpression of *BEM1* stimulates the kinase activity of the MAPK Fus3p (270) and suppresses the mating defect of a dominant negative STE4 mutant (234). These data suggest that Bem1p is involved not only in cross-linking the Ste5p-MAPK cascade complex to the cytoskeleton but also in transmitting signals to the MAPK cascade either directly or by facilitating its association with an upstream activator.

Sending Signals to the Nucleus: a Role for the MAPK Cascade

The pheromone-activated signaling pathway containing the Ste11p-Ste7p-Fus3p MAPK cascade is required for sending signals from the pheromone receptors in the plasma membrane to gene targets in the nucleus. There are no known second messengers relaying signals on the pathway. Therefore, some protein or protein complex must leave the cytoplasm and move across the nuclear membrane. In animal cells, MAPK moves from the cytoplasm into the nucleus following stimulation by growth factor (58). This movement involves dissociation of MAPK from its cytoplasmic complex with MEK (135). MEK appears to be in the cytoplasm and to remain there after growth factor treatment (522). However, more recent experiments suggest that MEK can also be induced to move from the cytoplasm to the nucleus following growth factor stimulation if its nuclear export signal (134) and catalytic site are inactivated by mutation (191). Disruption of the nuclear export signal in MEK strongly stimulates MEK-dependent morphological changes and malignant transformation (133). Thus, the apparent cytoplasmic localization of MEK in growth factor-stimulated cells may reflect transient nuclear entry followed by rapid export from the nucleus (133, 191). A leucine-rich sequence near the NH_2 terminus of MEK acts as the nuclear export signal (134); it is interesting that the yeast MEK Ste7p has a very similar sequence near its NH_2 terminus.

In the case of the yeast pheromone response pathway, it is still a mystery how the signal actually gets to the nucleus. Of the proteins on the MAPK cascade, the MAPK Fus3p appears to be present in the cytoplasm and nucleus (62). The MAPK Kss1p of the filamentation-invasion pathway (see below) is mostly in the nucleus (271). These MAPK locations change

little after pheromone treatment. Due to their apparent low abundance, the locations of Ste11p and Ste7p in the cell have been more difficult to determine and are not known with certainty at present.

Ste5p does seem to change location after pheromone treatment, although whether nuclear entry of Ste5p is required for signaling has not yet been determined. At different times and under different conditions, Ste5p is alternatively found at or near the plasma membrane, in the cytoplasm, or in the nucleus. Microscopic analysis shows Ste5p to be present in both the cytoplasm and the nucleus in vegetatively growing cells (283). After pheromone treatment, Ste5p moves from the nucleus to the cytoplasm and becomes associated with the plasma membrane in mating projections (97, 283). Interaction of Ste5p with Ste4p is required for the association of Ste5p with the plasma membrane (97). The association of Ste5p with the plasma membrane appears to be a critical step in signal transduction, because fusion of membrane-targeting signals to Ste5p induces activation of pheromone responses in the absence of added pheromone (384). A striking result is that Ste5p with an NH_2-terminal truncation removing the Gβ-binding domain is nonfunctional unless fused to membrane-targeting signals (384). Thus, plasma membrane localization of Ste5p is sufficient for signaling.

Ste5p localizes to the nucleus when untethered from Gβ (97, 283). Thus, Ste5p may be part of the signaling machinery that shuttles signals to the nucleus, perhaps released from Ste4p in pheromone-activated cells. It should, however, be pointed out that nuclear localization of Ste5p is not sufficient for signaling (97, 283). In addition, the situation may not be as simple as a single protein or protein complex shuttling signals to the nucleus: there may be multiple mechanisms acting in parallel. Deletion of the MEK gene *STE7* enhances Ste5p-Ste5p interaction in the two-hybrid system, suggesting that Ste7p-Ste5p and Ste5p-Ste5p complex formation might be mutually exclusive, i.e., that Ste5p dimerization might lead to Ste7p ejection (505). Ste5p preferentially interacts with the underphosphorylated, preactivated form of Ste7p, suggesting that phosphorylation of Ste7p might induce its release from the complex with Ste5p (61). Perhaps Ste7p, like the animal cell MEK (191), also carries signals to the nucleus. The tight complex formed between Ste7p and Fus3p (19, 21) suggests that instead of individual kinases, a complex of MEKK and MAPK may be the molecular species that carries signals to the nucleus. Movement of a protein or protein complex from the cytoplasm to the nucleus will require its dissociation from other cytoplasmic proteins. This may require more than one regulatory event or cooperative changes in protein conformation, especially in the case of ternary or higher-order complexes, where a protein must dissociate from more than one binding partner before it can break free of the complex (372).

Activation of transcription. Among the many aspects of the mating pathway that have been investigated so far, its regulation of transcription is fairly well understood. Pheromone stimulation activates the transcription of many different genes. Among the products of these genes are proteins that activate (e.g., Fus3p [113]) or inhibit (e.g., Msg5p [104, 519]) signaling on the pheromone response pathway and proteins needed for cell fusion (e.g., Fus1p [303, 466]), nuclear fusion (e.g., Kar4p [228]), and other mating-related functions. What these genes have in common is that they contain in their promoter region repeats of a pheromone response element (PRE) that is necessary and sufficient for pheromone regulated transcription (156, 222). The MAPK cascade mediates pheromone induction of transcription of PRE-containing genes through phosphorylation and activation of at least three nuclear proteins: Dig1p (68) (also called Rst1p [457]), Dig2p (68) (also called Rst2p [457]), and Ste12p (441).

Ste12p is a transcription factor containing separate domains for binding to the PRE, activation of transcription, and repression of transcription (209, 373, 441). Dig1p and Dig2p are related proteins with overlapping function that act as negative regulators of Ste12p function (68, 457). While Dig1p and Dig2p were originally thought to have a function specific for the invasion response (68), a second study demonstrated that Dig1p and Dig2p together repress the transcription of pheromone responsive genes (457). The increased expression of *FUS1*-lacZ in a *dig1*Δ *dig2*Δ strain requires Ste12p, arguing that Ste12p is the target of the repression (457). In contrast to the *dig1*Δ *dig2*Δ double mutant, single deletions of *DIG1* or *DIG2* have no obvious phenotype, suggesting that Dig1p and Dig2p perform a redundant function in cells (68, 457).

In an unstimulated cell, Dig1p and Dig2p appear to form a complex containing Fus3p (or Kss1p) and Ste12p (68, 373, 457). Pheromone stimulation increases Fus3p-dependent phosphorylation of Dig1p, Dig2p, and Ste12p (114, 457) and induces the release of Ste12p from the complex. Both Dig1p and Dig2p interact in the two-hybrid system with the transcriptional repressor domain of Ste12p (373). Putative MAPK phosphorylation sites in the Dig1p- and Dig2p-interacting domain of Ste12p are not required for Ste12p regulation (373). Thus, although the MAPK Fus3p phosphorylates Ste12p (114, 178), the function of this covalent modification remains unclear. Perhaps Ste12p release requires Dig1p and Dig2p phosphorylation, but this has not been tested yet. Pheromone treatment does not appear to alter Ste12p-DNA interaction but stimulates the activity of the transcriptional activation domain Ste12p (441). Freeing Ste12p from its association with the negative regulators Dig1p and Dig2p is therefore predicted to allow Ste12p to interact with other proteins of the transcription machinery and thereby activate transcription.

Induction of Cell Cycle Arrest

The MAPK pathway plays another important role in mediating cell cycle arrest in response to pheromone (494). Conjugation of two haploid mating partners is accompanied by the synchronization of the cell cycles of the two cells such that they both contribute 1N content of DNA to the zygote product of their union. Thus, mating pheromone-treated cells arrest at a position in the cell cycle prior to bud formation and initiation of DNA synthesis: they arrest as unbudded cells with a 1N DNA content. Growth of the G_1-arrested cell is not inhibited but redirected into the formation of mating projections. This pheromone-induced cell cycle arrest in G_1 involves signaling through the MAPK cascade (112, 113, 132, 469) and the cell cycle inhibitor Far1p (55, 141, 469).

To explain the mechanism of cell cycle arrest and how the MAPK pathway is involved, we first review the mechanisms that regulate cell cycle progression at the G_1/S transition in yeast (331). Formation of a bud, initiation of DNA synthesis, and duplication of the spindle pole body mark the progression of a yeast cell into S phase, past a G_1/S transition point called START. These post-START events require the activation of cyclin–cyclin-dependent kinase complexes consisting of the kinase Cdc28p and one of three G_1 cyclins: Cln1p, Cln2p, or Cln3p. An active G_1 cyclin–Cdc28p complex is needed to induce the degradation of a cyclin-dependent kinase inhibitor that is specific for B-type cyclin–Cdc28p complexes (413). This protein inhibitor, called Sic1p (344) (also called Sbd25p [106]), blocks the activity of Cdc28p in complex with the B-type cyclins Clb5p and Clb6p but not the activity of G_1 cyclin–Cdc28p

complexes. The B-type cyclin–Cdc28p complex, freed of its inhibitor protein, activates DNA replication (414). The mechanism responsible for activation of bud initiation by the G_1 cyclin–Cdc28p complex is independent of Sic1p (408, 468).

Cell cycle arrest by mating pheromone involves Far1p-dependent (367) and Far1p-independent processes (468). Far1p expression is normally restricted to the G_1 phase (305) by mechanisms of cell cycle-dependent transcription and protein turnover (167, 306, 347). Results from early studies indicated that Far1p is a cyclin-dependent kinase inhibitor that inhibits the activity of G_1 cyclin–Cdc28p complexes, but not that of B-type cyclin–Cdc28p complexes (196, 368). However, a more recent study could not detect a pheromone-induced reduction in the activity of the Cln2p-associated Cdc28p kinase, even though these complexes retain Far1p (141). Nevertheless, Far1p is required for pheromone-induced inhibition of G_1 cyclin–Cdc28p-dependent responses such as the expression of *CLN1* and *CLN2* (472). The MAPK Fus3p (but not Kss1p) is also required for cell cycle arrest in response to mating pheromone (112, 113). The functions of Fus3p and Far1p are linked, because pheromone induces the Fus3p-catalyzed phosphorylation of Far1p (56, 119, 141, 367, 469). G_1 cyclin–Cdc28p also phosphorylates Far1p (167, 367, 469) and thereby stimulates its degradation by a ubiquitin-dependent mechanism (167). Fus3p-catalyzed phosphorylation appears to have the opposite effect of stabilizing the Far1p protein (unpublished results cited in reference 167).

Far1p is a bifunctional molecule, required not only for cell cycle arrest but also for chemotropism (107, 473). This latter function is not connected to the function of the MAPK Fus3p or to that of the rest of the MAPK cascade (410). The way in which these two functions of Far1p are coordinated is not yet clear. Interestingly, the mechanism by which Far1p mediates cell cycle arrest is also not well understood at present (141).

The effects of pheromone on the cell cycle may be more complex than altering the activity of Far1p. In the absence of Cln1p, Cln2p, and the cyclin-dependent kinase inhibitor Sic1p, pheromone induces a Far1p-independent arrest of the cell cycle (468). In cells that have reduced activity of the Cln class of cyclin, another type of cyclin–cyclin-dependent kinase complex containing the Cdc28p-related protein Pho85p becomes critical for cell cycle progression (121, 308). The mRNA level for one of the Pho85p-associated cyclins, Pcl1p, is rapidly down-regulated by pheromone treatment (309) with a time course similar to that of the pheromone-induced decrease in Cln1p and Cln2p mRNA (495). Perhaps the Far1p-independent cell cycle arrest induced by pheromone treatment in *cln1*Δ *cln2*Δ *sic1*Δ cells (468) reflects parallel regulation of the Pho85p kinase through transcriptional control of expression of the Pcl1p cyclin associated with Pho85p. The Far1p-Cdc28p paradigm also suggests that the Pho85p inhibitor Pho81p (409) might be a target of regulation by the pheromone pathway. However, Pho85p interacts with many different cyclins, and its physiological functions appear to be very complex (9). For example, although Pcl1p and Pcl9p mRNAs are decreased by pheromone treatment, other Pho85p cyclins show no change or an increase in mRNA expression after addition of pheromone (309). Finally, the role of the MAPK pathway in the Far1p-independent cell cycle arrest by pheromone has not yet been determined.

As discussed above, the pheromone response pathway regulates the cell cycle but the converse is also true. For example, the basal level of protein kinase activity of MEK Ste7p and MAPK Fus3p fluctuates during the cell cycle, reaching a peak in early G_1 (481). The activity of the MAPK cascade—high in early G_1 and low in late G_1—correlates well with the amount of mRNA for different pheromone-dependent genes (346, 347, 517). These molecular changes in the absence of pheromone may allow the cell to be maximally responsive to pheromone in early G_1, a cell cycle position close to the pheromone arrest point in late G_1. The cell cycle regulation of the MAPK cascade and pheromone-dependent genes appears to be mediated through the G_1 cyclins Cln1p and Cln2p, cyclins that reach their peak expression level in late G_1 (392). Hence, overexpression of *CLN2* represses the mating pathway (346, 481). Analyses of various mutants that either allow the Cln2p repression or block its effect suggest that the target of Cln2p repression is downstream of the Gβ Ste4p. The MEKK Ste11p or one of the proteins involved in activating the MAPK cascade are the current candidates for the target of Cln2p repression (481). Another potential connection between Cln2p and the mating pathway is at the level of the MAPK substrates Dig1p and Dig2p, repressors of the transcription factor Ste12p (68, 457). Cln1p and Cln2p each show specific interactions with Dig1p and Dig2p in the two-hybrid system (457). While the functional significance of this interaction has not yet been determined, it is tempting to speculate that positive regulation of Dig1p and Dig2p by Cln2p-Cdc28p or Cln1p-Cdc28p might repress Ste12p and shut off the pheromone response, thereby enhancing recovery. In summary, there is a reciprocal relationship between the activities of the pheromone response pathway and the G_1 cyclin-Cdc28p complex, regulator of the G_1/S cell cycle transition, with each inhibiting the other. This situation allows the cell to make a clean switch from one function to the other, from budding to mating or vice versa.

Time and the MAPK Cascade

Time is an important parameter when one considers the physiological and molecular properties of a signaling pathway like a MAPK cascade. For responding to environmental changes or a potential sexual partner, the rapidity of signaling in a pathway has tremendous selective advantage. Fus3p becomes phosphorylated and active within 1 to 2 min after α-factor treatment (17, 62, 142). Other yeast MAPK pathways (discussed below) show a similar speed of response to stimuli. Another time-related factor is the relationship between the signal duration (e.g., pheromone) and the output response generated by the cell. Short-term activation of the MAPK pathway (~1 h) is sufficient to activate transcriptional responses to pheromone, while sustained activation (~3 h) is needed for cell cycle arrest (77). In a different system, the PC12 neuronal cell line, sustained activation of a MAPK cascade is required to induce differentiation and cessation of cell division. Transient activation of the cascade leads instead to increased cell proliferation (291).

Another time-related factor is the important function of turning off an activated pathway, allowing a cell to adjust to changing levels of an external stimulus. There are multiple mechanisms for down-regulating an activated mating-pheromone pathway, and attenuation of signaling on the MAPK cascade is part of the story. Following pheromone treatment, Ste7p activates the downstream MAPK Fus3p by inducing its phosphorylation (19, 114, 119, 271). However, the MAPKs Fus3p (119) and Kss1p (19) also phosphorylate the upstream MEK Ste7p; this phosphorylation appears to be part of a negative-feedback mechanism to shut off the MEK (142, 271, 526). Fus3p also phosphorylates the Ste5p scaffold protein (114), but the function of this modification is unknown. Several phosphatases act on the MAPK Fus3p: the dual-specificity phosphatase Msg5p (104, 519) and the tyrosine phosphatases Ptp2p and Ptp3p (519). The basal level of Fus3p phosphory-

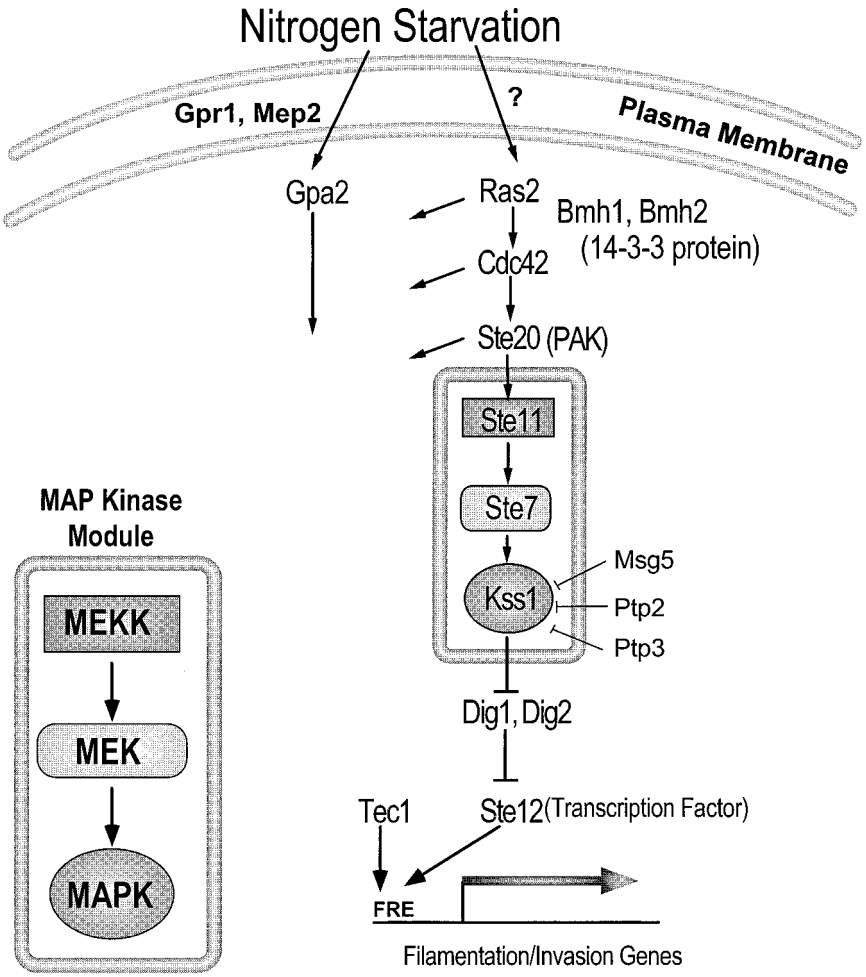

FIG. 3. Filamentation-invasion pathway of *S. cerevisiae*. Symbols are as described in the legend to Fig. 2. See the text for details of signal transduction between different proteins on the pathway.

lation is controlled mainly by the Ptp3p phosphatase (519). Pheromone treatment induces the expression of Msg5p (104), which then acts together with Ptp3p to inactivate Fus3p (519). Expression of *PTP2* and *PTP3* is not altered by pheromone treatment (519). Thus, deletion of these phosphatases delays the rate of recovery of pheromone-treated cells from cell cycle arrest whereas phosphatase overexpression speeds recovery. The location of these phosphatases in yeast is not known, but in animal cells similar phosphatases are localized to the nucleus (207).

FILAMENTATION-INVASION PATHWAY

Under specific culture conditions, diploid yeast will undergo a dimorphic switch and differentiate to form pseudohyphae, growing as filaments of extended and connected cells to form rough-edged colonies that invade solid medium. The physiological and genetic conditions necessary for this differentiation response have only recently been investigated. Starvation for nitrogen appears to induce the response (147), but other environmental factors may be important (260). Only a subset of commonly used laboratory strains have the right complement of genes to perform the switch (147, 258). The pseudohyphal response requires the cells to be diploid, although haploid strains can be induced to invade solid medium (395). The pseudohyphal response of diploid cells is characterized by changes in bud site selection from bipolar to unipolar, cell elongation, and invasive growth, each of which can be separated by mutation (323). This switch in cell properties from the "yeast" state to the pseudohyphal state probably involves multiple signaling pathways (31, 146, 147, 223, 264), one of which is very similar to the pheromone response pathway (257, 278, 395). The other pathways are not well defined at this writing, but at least one pathway appears to contain a Gα subunit encoded by *GPA2* (223, 264). Gpa2p appears to act in the same pathway as a G-protein-coupled, seven-transmembrane receptor encoded by *GPR1* (503), the Mep2p ammonium permease gene (262), and a downstream protein kinase encoded by *SCH9* (460, 503). Although the interactions between upstream components in the two pathways are not fully resolved, here the term filamentation-invasion pathway will be used for the former pathway that contains a MAPK cascade similar to that of the pheromone response pathway.

The filamentation-invasion pathway (Fig. 3) contains a MAPK cascade (257, 395) that mediates signal transduction from two small GTP binding proteins, Ras2p (147, 324) and Cdc42p (324). Signaling from Ras2p requires the 14-3-3 proteins Bmh1p and Bmh2p (145, 396). Cdc42p acts downstream of Ras2p (324) and is required for the function of the PAK Ste20p in the filamentation-invasion pathway (239, 369).

Cdc42p-Ste20p then transmits signal to the MAPK cascade. Like the pheromone response pathway, this cascade contains the MEKK Ste11p and the MEK Ste7p. However, the MAPK for the filamentation-invasion pathway is Kss1p (69, 278), in place of Fus3p (278). Also, the pheromone response pathway has Ste5p as a scaffold for the MAPK cascade (61, 218, 287, 383) while a MAPK cascade scaffold protein for the filamentation-invasion pathway has yet to be uncovered.

The filamentation-invasion pathway, like the pheromone response pathway, regulates transcription. Only two promoters have so far been identified as targets of the filamentation-invasion pathway: an upstream activating sequence in the Ty1 transposon (25, 276) and the promoter of the *TEC1* gene (276). The filamentation-invasion pathway-responsive, *cis*-acting regulatory sequences in these promoters are related to those in pheromone-regulated genes. Both types of regulatory sequences contain a PRE (156, 222), the binding site for the transcription factor Ste12p (105). Promoters regulated by the filamentation-invasion pathway have one copy of a PRE in close proximity to a binding site for a second transcription factor called Tec1p (25, 276). The regulatory DNA sequence containing both Ste12p and Tec1p binding sites has been termed a filamentation and invasion responsive element (FRE) (276). An FRE is both necessary and sufficient for transcriptional regulation by upstream activating signals in the filamentation-invasion pathway (276, 324). Both Ste12p (257, 395) and Tec1p (143, 323) are required for the pseudohyphal response. The *TEC1* promoter has an FRE, providing a positive-feedback mechanism for up-regulation of Tec1p in inducing the pseudohyphal response (276).

Dig1p and Dig2p act as negative regulators of Ste12p function in not only the pheromone response pathway but also the filamentation-invasion pathway (68, 457). Thus, *dig1*Δ *dig2*Δ cells show constitutive activation of the invasive growth response normally mediated by the filamentation-invasion pathway (68, 457).

One MAPK—One Pathway

Kss1p is the MAPK for the filamentation-invasion pathway (69, 277, 278). Historically, the observation that formation of pseudohyphae is not blocked by deleting any or all of the MAPKs in yeast led to an initial hypothesis that the filamentation-invasion pathway does not use a MAPK for signaling (257). For example, cells with or without the MAPK Kss1p show diploid pseudohyphal development on low-nitrogen medium, haploid invasive growth, and expression of FRE-*lacZ* (69, 257, 278, 395). However, cells with an inactivated Kss1p (with *STE7* deleted or expressing a nonphosphorylatable mutant Kss1p in a *kss1*Δ background) do not undergo pseudohyphal development or haploid invasive growth and have reduced FRE-*lacZ* expression (20, 69, 278). These findings indicate that the unactivated form of the Kss1p kinase inhibits the pseudohyphal response. The haploid invasive growth response is inhibited not only by Kss1p but also by Fus3p (69, 278).

Induction of the pseudohyphal response by the MEK Ste7p appears to involve two effects. Ste7p-catalyzed phosphorylation of Kss1p relieves inhibition of the pseudohyphal response by Kss1p. Expression of wild-type Kss1p or a catalytically inactive but phosphorylatable mutant of Kss1p allows a *kss1*Δ *fus3*Δ strain to show invasive growth and normal levels of expression of FRE-*lacZ* (20). In contrast, a nonphosphorylatable Kss1p does not allow these responses (20, 69, 278). The mechanism by which nonphosphorylated Kss1p inhibits invasive growth and FRE-dependent transcription appears to be mediated by binding of the unactivated MAPK to the transcription factor Ste12p. For example, a mutant of Kss1p that binds normally to Ste7p and to the Ste12p-repressors Dig1p and Dig2p but not to Ste12p was isolated. This mutant Kss1p can no longer inhibit the pseudohyphal response (20).

Ste7p-catalyzed phosphorylation of Kss1p not only removes a repressor (unphosphorylated Kss1p) but also appears to generate an activator (phosphorylated Kss1p). This dual role of Kss1p can be appreciated by comparing wild-type and *kss1*Δ strains. Although cells lacking (the repressor) Kss1p show some invasiveness and expression of FRE-*lacZ*, the levels of each are significantly lower than that observed for *KSS1*$^+$ cells (69, 278). Expression of hyperactive forms of either the MEKK Ste11p or the MEK Ste7p induces a strong pseudohyphal response and greatly increased FRE-*lacZ* expression (257, 276, 278). Cells with *KSS1* deleted show no response to expression of these hypermorphic mutants (278), providing further support for the idea that Kss1p in its phosphorylated, active state is a positive regulator of the pseudohyphal response.

Kss1p has also been proposed to be part of the pheromone response pathway MAPK cascade. Kss1p, in the absence of Fus3p, allows near-wild-type levels of mating (112, 142), suggesting that Kss1p may also play a part in signaling by the pheromone response pathway. A *fus3*Δ *kss1*Δ strain is thus completely sterile. Further support for Kss1p as a mediator of mating pheromone responses is that pheromone treatment increases Kss1p kinase activity (19), although the fold increase is much lower than that for pheromone stimulation of Fus3p kinase activity (114). Furthermore, Kss1p interacts in the two-hybrid system with the pheromone pathway scaffold protein Ste5p (61), although whether this interaction is mediated through the MEK Ste7p was not tested.

However, it has been recently argued that Kss1p is not normally part of the pheromone response pathway and fills that role only when Fus3p has been deleted. Rather, it was proposed on the basis of several observations that Fus3p is the MAPK for the pheromone response pathway (277, 278), just as Kss1p is the MAPK for the filamentation-invasion pathway (69, 278). Kss1p cannot fully cover for the loss of Fus3p. For example, pheromone-induced cell cycle arrest requires Fus3p and Kss1p cannot mediate this response (112). As mentioned above, pheromone does increase Kss1p kinase activity but the increase is much lower than that for the Fus3p kinase. Pheromone effects on Kss1p kinase activity were also tested under conditions of Kss1p overexpression (19), in which Kss1p could artifactually compete with Fus3p.

Deletion of *FUS3* may thus allow Kss1p to perform new functions; e.g., pheromone induces a Kss1p-dependent increase in FRE-*lacZ* expression but only in *fus3*Δ cells (278). A *fus3*Δ strain shows increased haploid invasive growth; *kss1*Δ or *ste4*Δ suppresses this phenotype. Haploid invasive growth of a wild-type *FUS3* strain is not inhibited by *ste4*Δ (278). These observations suggest that in the absence of Fus3p, Ste4p inappropriately signals to Kss1p and therefore activates FRE-dependent transcription of invasive growth genes. One mechanism to explain the *fus3*Δ phenotype is that the absence of Fus3p allows Kss1p to bind to the MAPK binding site on Ste5p and receive signals from pheromone. The observation that a strain expressing a catalytically inactive mutant Fus3p in a *fus3*Δ strain is more sterile than a *fus3*Δ strain (278) is consistent with this possibility. The inactive Fus3p mutant had no effect when expressed in a wild-type *FUS3* strain (278), showing that the mutant is not acting as a dominant negative mutant to Fus3p and, by extension, to Kss1p. Although many of these data support a model in which Fus3p is the MAPK for the pheromone response pathway, additional experimental tests

are needed to fully resolve this point. For example, it is important to know whether addition of mating pheromone induces the activation of Kss1p phosphorylation or kinase activity with similar kinetics to the observed activation of Fus3p, particularly under conditions where both proteins are present at wild-type expression levels. In addition, it is important to know whether Kss1p is physically associated with Ste5p in cells under conditions of normal expression levels for both proteins.

Signaling Proteins Shared by two MAPK Pathways

Yeast cells use the same signaling proteins (Ste20p, Ste11p, Ste7p, and Ste12p) in two different pathways that receive different input signals and generate different outputs. Pheromone induces mating, and nitrogen starvation induces filamentation and invasion. Three factors are important in matching input signal to output response by using the same signaling proteins for the central part of two different pathways. Cell-type-specific gene expression is one such factor. To respond to mating pheromone, cells need receptors for the pheromone (Ste2p and Ste3p) plus a G protein (Gpa1p-Ste4p-Ste18p), a MAPK cascade scaffold protein (Ste5p) to transmit the signal from the receptors to the MAPK cascade, and a MAPK (Fus3p) to induce cell cycle arrest. Diploid cells do not express these components and therefore cannot respond to mating pheromone (113, 163, 235, 326, 366, 442, 486). However, haploid cells can activate either a pheromone response pathway or a filamentation-invasion pathway (395). A second factor important for determining pathway specificity is a protein complex that allows specific input signals to the MAPK cascade and then to the transcription factor. One pathway-specific protein complex has been identified for the pheromone response pathway (e.g., the Ste4p-Ste5p-MAPK cascade) but the corresponding complex for the filamentation-invasion pathway is unknown. How pathway specificity is generated at the steps involving the PAK Ste20p and the transcription factor Ste12p, respectively, has not yet been determined. The final factor important for generating specificity is input from one or more additional pathways, a critical factor for the filamentation-invasion pathway (146, 223, 259, 264). Although the molecular details of transcriptional regulation during the pseudohyphal response are sketchy at present, it seems reasonable to expect that the combination of signals from different pathways dictates which genes to turn on and which to keep off.

Slow Responses to MAPK Pathways

At first glance, a pathway with a MAPK cascade appears to be selected for speed, responding rapidly to an environmental stimulus. Proteins are complexed so that there are few steps at which a protein must diffuse randomly through the cell to find the next signaling protein in the pathway. The pheromone response pathway (142), the cell integrity pathway (89), and the HOG pathway (44) all can activate their MAPKs in minutes after initial stimulus. The large-scale cellular responses, e.g., cell adhesion and fusion during mating, to pathway activation are of course very much slower. Nevertheless, the cellular responses to these pathways still occur within one cell generation, approximately 1.5 to 3 h.

The filamentation-invasion pathway and the responses it mediates seem much slower by comparison. Growth in medium that contains limiting amounts of nitrogen activates this pathway (324) and elicits the pseudohyphal response in diploid cells (147). Depletion of cellular nitrogen is likely to present a rather slow, graded stimulus rather than a rapid, step-like stimulus like a decrease in osmolarity or addition of mating pheromone. Typical responses such as filamentous growth and invasion of agar reflect the concerted activity of many cells (147, 221, 395). Expression of the FRE-*lacZ* reporter for the filamentation-invasion pathway is usually assayed after growing yeast strains for many generations (324). Thus, one could view the filamentation-invasion pathway as a potentially fast pathway mediating slow responses to a slow signal. Whether this pathway can react quickly, or even needs to do so, remains to be determined. It could be that the filamentation-invasion pathway allows for a slow increase in signaling, integrating many different inputs (e.g., nitrogen starvation, carbon starvation, or a change in the surrounding physical environment) until some threshold is reached and a switch is activated.

One real gap in our understanding of the filamentation-invasion pathway is the nature of the true activating physiological signal(s) for this pathway. More genetic and physiological analysis of the nitrogen limitation condition of the cell is needed to determine what aspect of nitrogen metabolism more directly activates the MAPK cascade and its downstream gene targets in diploids. Formation of cellular filaments by haploid cells does not require nitrogen limitation per se but appears to be triggered instead by nutrient limitation (395). The physical nature of the growth medium could also play a role in activating the pseudohyphal response (260). Dimorphic switching of bacteria to a hyperflagellated, swarming-motility cell type is induced by changes in the agar support, i.e., the physical properties of the growth medium (162). Certain fungi appear to be capable of sensing external mechanical stimuli (175). How direct is the effect of nutrient limitation on the filamentation-invasion pathway? Are new proteins expressed that activate the pathway, or do preexisting proteins mediate pathway activation? Although Ste20p, Ste11p, Ste7p, and Kss1p appear to be constitutively expressed in haploid and diploid cells, other, as-yet-uncharacterized activators of the MAPK pathway might be expressed in response to nutrient deprivation. There is precedent for this type of mechanism. For example, components of the spore wall assembly pathway (see below) are expressed during the time preceding the events they regulate (131, 220).

CELL INTEGRITY PATHWAY

A second MAPK cascade is found in budding yeast as part of the cell integrity pathway (Fig. 4). This pathway mediates cell cycle-regulated cell wall synthesis and responds to different signals including cell cycle regulation, growth temperature, changes in external osmolarity, and mating pheromone. Signaling proteins on the pathway include the GTP binding protein Rho1p (202, 340), the protein kinase C homologue Pkc1p (250), the MEKK Bck1p (242) (also called Slk1p [73]), the redundant pair of MEKs Mkk1p and Mkk2p (190), the MAPK Slt2p (465) (also called Mpk1p [241]), and the transcription factor targets Rlm1p (103, 483, 484) and SBF (275), the latter being composed of the polypeptides Swi4p and Swi6p (101, 211). There are probably many branches onto and off this pathway. For example, Rho1p interacts with and regulates more proteins than Pkc1p does (109, 170, 185, 214, 272, 300, 386) and Pkc1p regulates more than just the MAPK cascade (177, 242). Membrane proteins that potentially provide input signals to the cell integrity pathway include Wsc1p (476) (also called Hsc77p [151]), Wsc2p (476), and Wsc3p (476). Of all the MAPK cascades in yeast, this pathway is the most similar to the classical mitogen-activated ERK1-ERK2 MAPK cascade in animal cells in functional tests of the pathway components (37, 241, 256, 507). Also, both the mammalian MAPK cascade and the yeast cell integrity pathway have the same general function

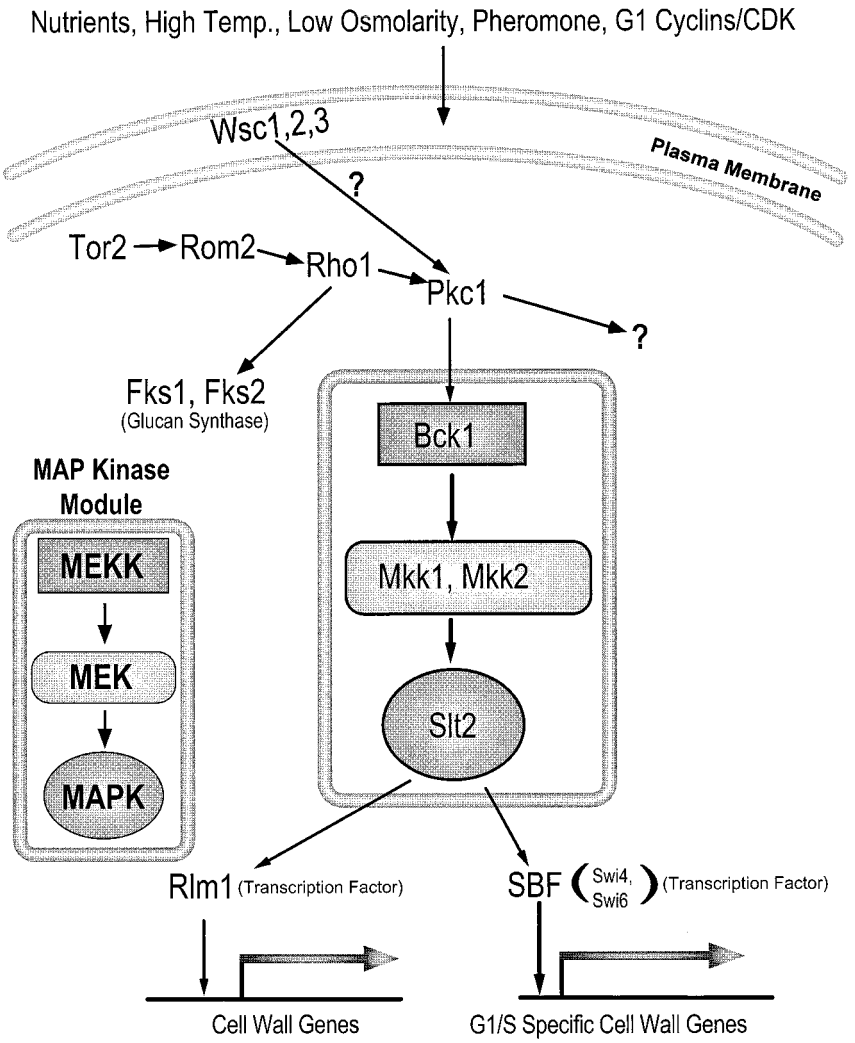

FIG. 4. Cell integrity pathway of *S. cerevisiae*. This pathway appears to be regulated by several different signals listed at the top: nutrients, temperature, osmolarity, pheromone, and cyclin-dependent kinase (CDK). Where these different upstream signals feed in to the pathway is currently unknown. Whether Rlm1p and SBF mediate pathway regulation of separate (as shown) or overlapping sets of genes is not known.

in their respective systems: to positively regulate growth and cell proliferation (151, 275, 288, 291, 518).

Activation of the Pathway

Cell cycle regulation. Formation of a new bud occurs at the G_1/S transition, after START (251, 252). This process requires the activity of the cyclin-dependent kinase Cdc28p in complexes with the G_1 cyclins encoded by *CLN2*, *CLN2*, and *CLN3*. Among the periodic events of the cell cycle that peak at the G_1/S transition is the transcription of cell wall genes (181). Several observations suggest that Cdc28p regulates the cell integrity pathway, which in turn induces cell wall gene expression. Increasing or decreasing the activity of Cdc28p in cells induces corresponding increases or decreases in the tyrosine phosphorylation and kinase activity of the MAPK Slt2p (288, 518). Mutations in the cell integrity pathway kinases show synthetic lethality with *cdc28* mutations (288, 302). Measurements of the amount of tyrosine phosphorylated Slt2p at different points in the cell cycle show a peak of phosphorylated Slt2p at the G_1/S transition (518). The time of maximum Slt2p phosphorylation thus correlates with the time of polarization of growth toward the bud tip (251). This peak of Slt2p phosphorylation correlates approximately in time with the peak in G_1 cyclin-Cdc28p activity and an increase in cell wall gene expression (181). PKC1 is required for cell cycle-dependent expression of a subset of cell wall genes (*FKS1*, *MNN1*, and *CSD1*) (181). A mechanism by which the cell integrity pathway regulates the expression of the cell wall genes is discussed below.

The mechanism by which G_1 cyclin-Cdc28p stimulates the cell integrity pathway is not known. One proposed mechanism (288) is based on the observation that Cdc28p induces at START the hydrolysis of phosphatidylcholine to choline phosphate and diacylglycerol. The increase in the amount of diacylglycerol is then proposed to activate Pkc1p similarly to the activation of mammalian cell protein kinase C by this lipid hydrolysis product (339). Mutants of Pkc1p defective in a putative diacylglycerol binding site cause a partial loss of Pkc1p function (192). However, attempts to show regulation of the cell integrity pathway in vitro or in vivo with diacylglycerol or the related phorbol esters have been unsuccessful (12, 89, 482). One caveat to in vitro studies showing a lack of effect of

diacylglycerol on Pkc1p is that the amount of Pkc1p that can be extracted from yeast in a soluble form is only about 5% of the total (12, 482). Whether the properties of this enzyme are representative of the total Pkc1p is unclear. In addition, the inability of diacylglycerol to activate Slt2p phosphorylation in vivo (89) could result from an inability of the lipid to pass the cell wall. The question whether Pkc1p is activated by the products of phospholipid hydrolysis is thus still open.

Heat stress activation. The cell integrity pathway is required both for growth of yeast at elevated temperatures (242, 250) (see below) and also for induced thermotolerance (201), i.e., the ability of cells to better survive severe heat shock if they are first exposed to mild heat shock. Not only is the MAPK cascade of this pathway required for growth and viability under heat stress conditions, but also the pathway is activated by heat stress. Increasing the growth temperature from 23 to 39°C induces the tyrosine phosphorylation of the MAPK Slt2p (201, 518) and increases its kinase activity (201). The change in Slt2p kinase activity is large (~100-fold), requires Pkc1p and the MEKK Bck1p, develops ~20 min after heat shock to its maximum at 30 min, and does not require new protein synthesis (201). The slowness of the response suggests that heat stress is not the primary signal for activation of the cell integrity pathway but instead reflects a physiological property of the cell affected by heat stress. Analysis of Slt2p tyrosine phosphorylation shows that raising the temperature increases the overall activity of the cell integrity pathway but has little effect on its periodicity during the cell cycle (518). After heat stress, Slt2p phosphorylation still peaks in late G_1 and early S phase, although the S-phase Slt2p phosphorylation appears to be increased relative to that observed at lower temperature (518).

The idea that the cell integrity pathway plays a physiological role in adapting to heat stress was suggested first by the phenotype of pathway mutants. Mutants lacking Pkc1p (247, 357), the MEKK Bck1p (242), the MEKs Mkk1p and Mkk2p (190), or the MAPK Slt2p (241, 302) are temperature sensitive, with the cells growing less well at 37°C than at room temperature. The temperature sensitivity of the deletion mutant strains suggests that the cell integrity pathway has a physiological function that is required for growth at higher temperature. The temperature sensitivity of the pathway mutants is correlated with increased cell lysis (247, 357), a defect caused by weaker cell walls (248, 399). This phenotype suggests the possibility (201) that increased growth temperature creates stress on the cell wall or underlying plasma membrane, which then activates the cell integrity pathway and thereby increases cell wall gene expression (181) and cell wall synthesis to relieve the stress. The way in which growth temperature puts stress on the plasma membrane or cell wall is not known. Increasing the osmolarity of the medium suppresses the temperature sensitivity and cell lysis defect of the cell integrity pathway mutants (73, 242, 247, 357). Increasing the external osmolarity is predicted to collapse the osmotic gradient across the cell membrane and reduce pressure on the cell wall.

Hypotonic stress activation. Growing yeast, like plants and other organisms with a cell wall, maintain an osmotic gradient (high inside) across their plasma membrane (35, 176) to drive the uptake of water needed for cell growth and create turgor pressure. Decreasing the external osmolarity is predicted to increase the magnitude of this osmotic gradient and turgor pressure, creating stress on the plasma membrane and cell wall. Thus, another signal that activates the cell integrity pathway in cells is hypotonic stress, a decrease in the osmolarity of the growth medium (89, 201). Hypotonic stress induces an increase in tyrosine phosphorylation and protein kinase activity of the cell integrity pathway MAPK Slt2p. This response requires Pkc1p, the MEKK Bck1p, and the MEKs Mkk1p and Mkk2p. This signaling response is likely to be physiologically relevant, because pathway genes are required for growth in low-osmolarity medium (247, 358). Thus, higher temperature and lower osmolarity are two environmental conditions which activate the cell integrity pathway and for which pathway function is required. One important difference between these two stimuli is the relative speed of the response of the cell integrity pathway. Decreasing osmolarity increases the phosphorylation of the MAPK Slt2p within seconds (89), whereas increasing temperature activates the pathway within tens of minutes (201). The speedy response to hypotonic stress suggests a more direct effect of this stimulus in activating the pathway.

Other data from a study on cell fusion during mating (371) support the idea that the magnitude of the osmotic gradient across the plasma membrane is a signal that regulates the cell integrity pathway. First, expression of a hyperactivated allele of *PKC1* blocks fusion of mating cells (371). Interestingly, the same phenotype is observed in cells lacking Fps1p (371), a glycerol transporter (268, 450). The *fps1*Δ cells have increased amounts of intracellular glycerol. Deletion of *GPD1*, encoding an enzyme needed for glycerol synthesis, suppresses the cell fusion defect of *fps1*Δ but not that induced by expression of the activated Pkc1p. These results are consistent with a model in which an elevated osmotic gradient (in *fps1*Δ cells) causes hyperactivation of the cell integrity pathway, which in turn blocks cell fusion (371). Deletion of *FPS1* causes no obvious growth phenotype but greatly enhances the temperature-sensitive, osmotic remedial cell lysis phenotype of an *slt2*Δ mutation (371). Thus, the cell integrity pathway appears to be needed for survival when the osmotic gradient is increased, i.e., under conditions of high turgor pressure.

Activation by mating pheromone. The cell integrity pathway is involved in different aspects of the mating response. Mutants lacking pathway genes are defective for pheromone-induced formation of mating projections (73), eventually losing viability with sustained pheromone treatment (118). As discussed below, pheromone treatment activates the cell integrity pathway and the timing of activation following pheromone treatment is closely correlated with the time of mating-projection formation. Mutations in Spa2p and Bni1p, proteins normally localized to the mating-projection tip, induce similar delays in both pathway activation and projection formation, suggesting that the two responses are linked in some way (50). Also, turning off the cell integrity pathway may be required for the fusion of two mating cells, because expression of an activated form of Pkc1p blocks cell fusion (371).

Additional evidence supporting a role for the cell integrity pathway in mating includes observations showing that pheromone treatment increases the tyrosine phosphorylation (518) and kinase activity (50, 118) of the MAPK Slt2p. Pheromone activation of Slt2p takes ~15 to 30 min and is inhibited in cells that either lack a functional Pkc1p or have MEKs Mkk1p and Mkk2p deleted (50, 518). One research group investigating pheromone activation of the cell integrity pathway found that the MEKK Bck1p is required (518), while a second group found that *bck1*Δ only partially blocked Slt2p activation, suggesting that a second MEKK may help mediate this response to pheromone (50). The part of the pheromone response pathway that is responsible for regulation of the cell integrity pathway is somewhat controversial. One group (518) found that *ste20*Δ but not *ste11*Δ or *ste12*Δ completely blocks pheromone activation of Slt2p phosphorylation, suggesting that Ste20p regulates the cell integrity pathway through a mechanism that does not involve the MAPK cascade of the pheromone response pathway. Interestingly, this group found that heat stress

still activates Slt2p phosphorylation in a *ste20*Δ strain, showing that heat stress and pheromone activation of the cell integrity pathway are mediated through separate upstream regulatory proteins (518). A second group (50), working with a different strain background (a *cdc28* mutant), observed that *ste12*Δ or a protein synthesis inhibitor completely blocks pheromone activation of Slt2p kinase activity. Their results suggest that pheromone activation of the cell integrity pathway may be mediated by proteins whose expression is induced by pheromone. Further investigation of the connection between these two pathways is required before these differences can be resolved.

Molecular mechanisms of pathway activation. Cell wall or plasma membrane stress, induced by heat shock or hypotonic medium, is a signal that must be transduced from the plasma membrane to Pkc1p and the downstream MAPK cascade. Candidates for plasma membrane signal transducers of this stress signal to the protein kinase cascade include Wsc1p (476) (also called Hcs77 [151]) and the related proteins Wsc2p, Wsc3p, and Wsc4p (476). As predicted from their amino acid sequences, these proteins have a single putative transmembrane domain with a cysteine-rich and serine/threonine-rich extracellular domain and an intracellular domain without significant homology to any other proteins. Wsc1p is localized to the plasma membrane of the cell (476). Heat stress-induced activation of the cell integrity pathway, measured as an increase in MAPK phosphorylation (476) or protein kinase activity (151), is greatly reduced in strains lacking Wsc proteins. The phenotype associated with *wsc1*Δ is similar to that of cell integrity pathway mutants: temperature-sensitive cell lysis that is suppressed by high-osmolarity medium (151, 476). The phenotype associated with a triple mutation, *wsc1*Δ *wsc2*Δ *wsc3*Δ, is stronger than that associated with any single mutation, suggesting that the Wsc-class proteins are related not just in amino acid sequence but also in function (476). The Wsc proteins are more likely to act in the cell integrity pathway rather than in a parallel pathway, because a *pkc1*Δ *wsc1*Δ *wsc2 wsc3*Δ strain is no more temperature sensitive than either a *pkc1*Δ strain or a *wsc1*Δ *wsc2*Δ *wsc3*Δ strain (476). The cell lysis defect of the *wsc1*Δ *wsc2*Δ *wsc3*Δ mutant is suppressed by overexpression of Rho1p and Pkc1p (151, 476). Whether the Wsc proteins are the stress sensors per se or are needed in a supporting role for signal transduction remains to be determined.

(i) Rho1p and the coordinated regulation of cell wall construction. Rho1p is a small GTP binding protein of the Rho subfamily of Ras-related proteins and is required for cell growth (274, 385). Important for the topic of this review is that Rho1p binds to and is required for the activity of Pkc1p in vivo (202, 340); Pkc1p in turn regulates the MAPK pathway. Although *RHO1* is an essential gene, a subset of temperature-sensitive *rho1* mutants is defective in heat stress-induced activation of Slt2p kinase activity (202). This type of *rho1* mutant also has a temperature-sensitive cell lysis defect that is suppressed by either increasing the osmolarity of the medium, expressing an activated mutant of Pkc1p, or overexpressing different downstream protein kinases of the cell integrity pathway (202, 340, 385). The GTP-bound form of Rho1p interacts preferentially over Rho1p-GDP with Pkc1p in the two-hybrid system (340) and in in vitro assays with recombinant protein (202). Rho1p in its GTP-bound form does not stimulate Pkc1p kinase activity in vitro but allows Pkc1p to be stimulated by the anionic phospholipid phosphatidylserine (202).

The exact role of Rho1p in the heat stress activation of the cell integrity pathway is still uncertain. Rho1p could transduce signal to Pkc1p. In this model, heat stress would induce a shift from Rho1p-GDP to Rho1p-GTP, which would then bind to Pkc1p and allow the protein kinase to be activated by phosphatidylserine or perhaps another anionic phospholipid. Phosphatidylinositol 4,5-diphosphate, an anionic phospholipid, is a possible Pkc1p activator (see below). Arguing against Rho1p as a transducer of signal to Pkc1p is the observation that heat stress does not induce a change in the amount of Pkc1p immunoprecipitated with Rho1p from cells (202). An alternative possibility is that Rho1p is required for signaling to take place but does not actually transduce signals to Pkc1p. Rho1p is not distributed randomly within the cell but is localized to regions of active cell growth such as the bud tip and neck region between the mother and daughter cells (509). Thus, Rho1p could function to localize Pkc1p in the cell so that Pkc1p can be properly regulated by nearby signal transduction proteins or other signaling molecules. A critical piece of information that is lacking is whether heat stress or other activating stimuli induce a shift from Rho1p-GDP to Rho1p-GTP on the same timescale as the observed activation of the cell integrity pathway. This measurement should be technically feasible. The effect of extracellular signals on Ras and Rho guanine nucleotide exchange has been measured for mammalian cells (233, 253). In one study on lymphoid cells (233), agonist activated an increase in guanine nucleotide exchange on Rho within seconds. Thus, the timescale of these reactions make them sufficiently fast to be considered as possible signaling intermediates in the hypotonic stress-induced activation of the yeast cell integrity pathway.

Another approach to determining whether Rho1p function is altered by heat stress or hypotonic shock is to examine the effect of these stimuli on the activity of the proteins that regulate Rho1p activity. These proteins include (i) the *ROM1*- and *ROM2*-encoded guanine nucleotide exchange factors (355, 405) that convert Rho1p-GDP to Rho1p-GTP, (ii) the *BEM2* and *SAC7*-encoded Rho1p-specific GAPs (208, 370, 405, 479) that convert Rho1p-GTP to Rho1p-GDP, and (iii) the *GDI1*-encoded rho GDP dissociation inhibitor (139, 213, 296) that binds the GDP-bound inactive form of the Rho1p and inhibits its activation. Rom2p, in particular, appears to be a potential upstream mediator of cell integrity pathway-activating signals. Cells with *ROM2* deleted have a temperature-sensitive growth phenotype that is suppressed by growth at high osmolarity or by overexpression of *RHO1* (355). Rom2p is localized to sites of polarized cell growth (284), the same place where Rho1p is found (509).

Going further upstream, Rom1p and Rom2p are regulated by the phosphatidylinositol kinase homologue Tor2p (405). Tor2p is thus another possible mediator of signaling to the cell integrity pathway. The Tor proteins, Tor1p and Tor2p (52, 166, 224, 263, 524), respond to nutrient availability and act as positive regulators of translation initiation and progression through G_1 (18, 98, 458). Tor2p has an additional function of promoting the organization of the actin cytoskeleton in G_1 phase (165, 406). Temperature-sensitive *tor2* mutants are suppressed by growth in high-osmolarity medium and by overexpression of *PKC1* (165), indicating a possible role for Tor2p in regulating the cell integrity pathway. Also, the connection between Tor proteins and nutrient sensing is intriguing because cells with the cell integrity pathway MEKK Bck1p deleted show a loss of cell viability in nutrient-poor medium, suggesting that this MAPK cascade may also be involved in nutrient sensing (75).

Rho1p has other cellular functions beside its role in cell integrity pathway function. The β-1,3 form of glucan is a major structural component of the yeast cell wall (351), and Rho1p is needed for its synthesis. The cell wall polysaccharide β-1,3-glucan is synthesized at the cell surface (420, 421) by a pair of differentially expressed glucan synthases, Fks1p and Fks2p (53,

108, 140, 186, 388). Rho1p is required in its GTP-bound form for activity of the plasma membrane-bound Fks1p (109, 300, 318, 386). Rho1p and Fks1p copurify from cells (109, 300, 386) and colocalize in the cell (386). The *RHO1* dependence of glucan synthase occurs even in a strain expressing a constitutively active, Rho1-independent Pkc1p mutant (109). Pkc1p is not detectable in Fks1p-Rho1p complexes (109), suggesting that Rho1p forms separate complexes with glucan synthase and Pkc1p, respectively. The cell integrity pathway is required for cell cycle-regulated expression of *FKS1* (181) and appears to regulate the expression of *FKS2*, a second β-1,3-glucan synthase gene (202). Rho1p therefore appears to regulate β-1,3-glucan synthesis at two levels, direct regulation of the enzyme itself and, through the MAPK cascade, regulation of expression of the enzyme.

Another mechanism by which Rho1p and the cell integrity pathway may regulate the construction of the cell wall is by regulating the delivery of another cell wall polysaccharide, β-1,6-glucan (210, 351), to the cell wall. Strains with *PKC1* deleted not only have less β-1,3-glucan in their cell wall but also have a smaller amount of β-1,6-glucan (399). In comparison to the longer β-1,3-glucan chains made at the cell surface (420, 421), the shorter chains of β-1,6-glucan are synthesized in internal membrane compartments (307, 398, 399). This glucan is secreted by exocytosis and is covalently cross-linked to β-1,3-glucan, mannoproteins, and chitin, the other major structural components of the cell wall (215). Cells lacking the MAPK Slt2p accumulate secretory vesicles in the bud (302), suggesting that the MAPK cascade is a positive regulator of exocytosis and delivery of β-1,6-glucan to the cell surface.

Rho1p-GTP (214) and other Rho family proteins, Cdc42p and Rho3p (124), bind to Bni1p, a cytoskeletal protein needed for proper bud site selection and rearrangement of the actin cytoskeleton during formation of mating projections. However, *rho1*Δ cells show normal localization of actin patches and cables but go on to lyse at the small-bud stage, suggesting a more restricted role in regulating cell wall synthesis (509). Cells with both of the Rho1p guanine nucleotide exchange factors, Rom1p and Rom2p, deleted have the same terminal cell morphology as *rho1*Δ cells (355). Thus, this lack of correlation between the *rho1* and *bni1* phenotypes suggests that the situation is more complex than Rho1p regulating Bni1p in some linear pathway.

Other potential signaling proteins show interaction with the cell integrity pathway but seem less likely to act as upstream regulators. One such example is Bro1p (337). Bro1p is similar to other signal transducers, including one involved in pH sensing in *Aspergillus* (333). A *bro1*Δ strain, like MAPK cascade mutants, has a temperature-sensitive cell lysis phenotype that is suppressed by growth in high-osmolarity medium (337). Overexpression of the MEKK Bck1p suppresses the temperature-sensitive phenotype caused by *bro1*Δ. Cell integrity pathway MAPK cascade mutants are sensitive to caffeine (73) and show a loss of cell viability in nutrient-poor medium (75): *bro1*Δ cells share this phenotype (337). However, some properties of MAPK cascade mutants, including synthetic lethality with *ppz1*,*2*Δ and *bck2*Δ (240) and defective shmoo formation (73), are not shared by *bro1*Δ (337). A deletion mutation of *BRO1* shows synthetic lethality with mutations in the cell integrity pathway (337). These data suggest that Bro1p acts in parallel with the MAPK cascade and is required for similar functions in the cell.

Stt4p is a phosphatidylinositol 4-kinase that is proposed to act either upstream of or in parallel to the cell integrity pathway (512, 513). The function of Stt4p is related (82) to that of Mss4p (513), a phosphatidylinositol 4-phosphate 5-kinase homologue (102), and the phosphoinositide-specific phospholipase C, Plc1p (129, 365, 511). Although certain isoforms of phosphoinositide-specific phospholipase C act upstream of protein kinase C in animal cells, the difference in phenotype between *pkc1*Δ (247, 250, 357) and *plc1*Δ (129, 365, 511) argues against a linear pathway in which Pkc1p is regulated by Plc1p. The phosphatidylinositol 4-kinase Stt4p, on the other hand, may regulate the cell integrity pathway or act in parallel. Deletion of *STT4* is lethal in all genetic backgrounds except one. The *stt4*Δ mutant in that background shows a reduced growth rate that, like cell integrity pathway mutants, is partially rescued by addition of 1 M sorbitol (82, 512). Cells lacking Stt4p, again like *pkc1* mutants, are also sensitive to the protein kinase C inhibitor staurospaurine, and overexpression of *PKC1* rescues the latter phenotype but does not rescue the slow-growth phenotype (512).

Interconnections of the Pathway

The cell integrity pathway MAPK cascade, together with the signaling proteins that input signals to the pathway and the targets of the cascade, must form specific complexes that allow rapid signaling and control cross talk with other pathways. A cell integrity pathway equivalent of the pheromone response pathway Ste5p, a scaffold protein that holds together the different protein kinases, has not yet been uncovered. Such a scaffold may not be required. The cell integrity pathway components are unique to that pathway, whereas the pheromone response pathway MEKK and MEK are shared with other pathways, suggesting the need for a scaffold protein to keep their functions isolated. By using the two-hybrid system, a series of interactions have been observed between kinases of the cell integrity pathway (358, 440). For example, Pkc1p interacts with the MEK Mkk1p independently of the MEKK Bck1p. The MEKK Bck1p interacts with Mkk1p and Mkk2p through the COOH terminus of the MEKs. Mkk1p and Mkk2p interact with the MAPK Slt2p. Interestingly, as in the HOG pathway (279, 378) (see below), the MEK of the cell integrity pathway appears to play a central role in organizing that MAPK cascade. Although there are important limitations of the two-hybrid system that counsel against overinterpreting its results (372), it is intriguing that Pkc1p shows a two-hybrid interaction with the MEK Mkk1p and no other protein kinase of the cascade (358). Spa2p and Sph1p are two nonessential proteins that localize to growing regions of the cell (14, 438); Spa2p is required for bud site selection and for formation of mating projections (144). Both of these proteins interact in the two-hybrid system with the MEKs Mkk1p and Mkk2p, through the NH_2-terminal nonkinase domain of the MEK (400, 422). Whether these interactions with Spa2p and Sph1p localize the MAPK cascade or mediate signal transmission to the protein kinases is not yet known. In this context, an interesting and potentially important effect of a *spa2*Δ mutation is that it delays pheromone induction of Slt2p kinase activity but blocks the later decline in activity of this MAPK (50). Without stimulation by pheromone, *spa2*Δ cells show increased Slt2p kinase activity and elevated phosphorylation of the Slt2p substrate Swi6p over that observed in *SPA2*$^+$ cells (422). Spa2p thus appears to act as a negative regulator of the cell integrity pathway, but the physiological meaning of this regulation remains mysterious.

Transcriptional Regulation

Several proteins have been identified as downstream substrates of the Slt2p MAPK. One of these, Rlm1p, a transcriptional regulatory protein, is discussed below. Two others,

Swi4p and Swi6p (275), together form the transcription factor SBF (10, 42, 382, 432). Swi4p is the DNA binding subunit and transcriptional activator of SBF and is required for normal expression of the G_1 cyclin genes *CLN1*, *CLN2*, *PCL1*, and *PCL2* at the G_1/S transition (79, 212, 308, 332, 348, 362, 448). Swi6p is more of a regulatory subunit, because loss of Swi6p leads to constitutive intermediate levels of *CLN1* and *CLN2* expression (101, 266). Cln1p and Cln2p are G_1 cyclins that complex with the cyclin-dependent kinase Cdc28p and thereby activate the G_1/S transition (78, 394). The complex of Pcl1p and Pcl2p with a different cyclin-dependent kinase, Pho85p, also promotes entry into S phase (121, 308). SBF is therefore a key regulator of the G_1/S transition.

The cell integrity pathway, like SBF, appears to be important for the G_1/S transition. One mechanism by which this pathway may promote this transition is through regulation of the SBF transcription factor. Phenotypic similarities between cell integrity pathway mutants and SBF mutants suggest this connection. For example, strains with mutations in *SLT2* or *PKC1* show a reduction in expression of several SBF-regulated genes expressed in late G_1 or early S phase, including the G_1 cyclin genes *PCL1* and *PCL2* (275) and several cell wall genes (181). In addition, the temperature-sensitive, osmosis-remedial cell lysis phenotype of a *swi4*Δ strain in some strain backgrounds (275, 348) is similar to that of *slt2*Δ strains. The cell integrity pathway appears to regulate SBF through Slt2p-catalyzed phosphorylation of the SBF subunits Swi4p and Swi6p (275). The amount of Swi6p phosphorylation in cells correlates well with the activity of Slt2p under a variety of conditions, e.g., changes in Slt2p expression level and growth temperature (275). This phosphorylation is likely to be the direct result of Slt2p-catalyzed phosphorylation because Slt2p is associated with SBF in cells and Slt2p phosphorylates the SBF subunits in vitro (275). Not all SBF-regulated genes require the MAPK cascade for proper expression; for example, *CLN1* and *CLN2* expression is unaltered by *slt2*Δ (275). Cell integrity pathway mutations are lethal when combined with *swi4*Δ (182), indicating that SBF and the MAPK cascade have some nonoverlapping functions.

SBF plays an important role in cell cycle progression at the G_1/S transition and is therefore the target of several regulatory pathways. SBF function is regulated by the G_1 cyclin Cln3p-Cdc28p kinase (100, 449). Because the cell integrity pathway is activated by Cdc28p (see above), it may be that this pathway mediates part of the cell cycle regulation of SBF activity. However, as discussed above, the MAPK cascade is required for expression of only a subset of SBF-regulated genes. Different DNA sequences appear to mediate SBF-dependent transcription (79, 362, 448), and so it seems reasonable to expect that SBF interacts with other, still unknown proteins. These proteins would help determine not only which subset of SBF targets is regulated by specific stimuli but also whether the gene expression is repressed or activated. The response to DNA damage illustrates this last point. DNA-damaging agents also induce the phosphorylation of Swi6p (174, 433), but the effects on SBF function are complex, inhibiting the SBF-dependent expression of *CLN1* and *CLN2* (433) and activating the SBF-dependent expression of a subset of the DNA damage-inducible genes (174). The DNA damage-induced phosphorylation of Swi6p appears to be mediated by the casein kinase I isoform Hrr25p (174). The way in which phosphorylation of SBF by Slt2p or other protein kinases affects its function is unknown. One possibility, translocation to the nucleus of Swi6p in late G_1, does not appear to be stimulated by phosphorylation but is instead activated by dephosphorylation (434).

Another target of the cell integrity pathway is Rlm1p, a member of the MADS box family of transcription factors (430). Rlm1p and the related yeast protein Smp1p are most similar to the mammalian MEF2 subfamily of MADS box proteins (515). This family of transcription factors generally form heterodimers with other transcription factors and are known to be MAPK targets in mammalian cells (159, 206) as well as in yeast cells (103, 483, 484). Overexpression of a hyperactivated cell integrity pathway MEK (Mkk1p) inhibits the growth of yeast. Mutations in *rlm1* were initially isolated as suppressors of the toxicity of activated Mkk1p overexpression (483); *rlm1* cells are also resistant to the growth inhibition induced by overexpression of an activated allele of *RHO1* (103). A similar strategy, in which the toxicity of pathway hyperactivation is used to isolate suppressors in pathway genes, has also been used with great success in studies of the HOG pathway (281) and a related pathway in fission yeast (312, 428).

Rlm1p appears to be a downstream substrate of the cell integrity pathway MAPK Slt2p. Rlm1p is a substrate for the MAPK in vitro and shows heat stress-induced, *SLT2*-dependent phosphorylation in vivo (484). Rlm1p also interacts with Slt2p in the two-hybrid system (483). Expression of a reporter construct consisting of a MEF2-consensus DNA target element fused to *lacZ* is both *RLM1* and *SLT2* dependent (103). The COOH-terminal two-thirds of Rlm1p is sufficient to mediate *SLT2*-dependent transcriptional induction. This was demonstrated (484) by replacing the NH_2-terminal, putative DNA binding domain of Rlm1p with that of LexA and expressing this fusion protein in a strain containing a LexA promoter-*lacZ* construct. Strong expression of the *lacZ*-encoded β-galactosidase was induced by the LexA protein-Rlm1pΔN fusion; mild heat stress induced expression by two- to threefold. Deletion of *SLT2* or *BCK1* reduced the expression to ~5% or less of control. Rlm1p has three functional domains: the NH_2-terminal MADS box DNA binding domain, a central domain that is the target for phosphorylation and regulation by the MAPK Slt2p, and a COOH-terminal region that is required for transcriptional activation (484).

Slt2p is not the only protein kinase to interact with Rlm1p. A two-hybrid screen for Rlm1p-interacting proteins identified Mlp1p (484), a putative protein kinase with strong sequence similarity to Slt2p and placed by sequence alignment analysis in the MAPK family (179). Mlp1p binds to the same region on Rlm1p as does Slt2p (484). Deletion of *MLP1* has no phenotype by itself except for its effect on the caffeine sensitivity of mutants with different cell integrity pathway mutations: *mlp1*Δ enhances the caffeine sensitivity of *slt2*Δ, and overexpression of Mlp1p suppresses the caffeine sensitivity of *bck1*Δ. This suppression of part of the *bck1*Δ phenotype requires Rlm1p, because overexpression of Mlp1p does not suppress the caffeine sensitivity of a *bck1*Δ *rlm1*Δ strain (484).

Let us divert our attention briefly and consider the structure of Mlp1p and its significance for MAPK function. Mlp1p is a bit unusual in that one of the two consensus phosphorylation sites, a conserved threonine, in the MAPK activation domain of Mpl1p is replaced with lysine and a consensus lysine in the catalytic site region is replaced by arginine. These substitutions have been introduced into other yeast MAPKs to make them inactive (142, 241, 271, 411)! The Lys in the active site is needed for phosphotransfer from ATP, and phosphorylation of the Thr (and Tyr) by a MEK is needed for binding protein substrate; phosphorylation of tyrosine is less important. Perhaps like Kss1p (69, 278), Mlp1p has a function in the inactive state and has been subject to selective pressures that favor a less functional protein kinase. The tyrosine in the MAPK activation domain of Mlp1p is required for suppression of the

caffeine sensitivity of *bck1*Δ, suggesting that the function of Mlp1p might be regulated by a MEK-catalyzed phosphorylation of this residue.

Genetic analysis of Rlm1p function makes it clear that there are two outputs from the cell integrity pathway MAPK cascade and that they involve different transcription factors and regulate different sets of genes involved in different cellular responses. Mutants lacking pathway kinases have a pleiotropic phenotype that includes temperature-sensitive growth (73, 242) and sensitivity to caffeine (73). The way caffeine affects cells is not known at the molecular level, but it enhances the cell lysis phenotype of *slt2* mutants (292). Caffeine probably affects some aspect of cell wall synthesis, because a large subset of other mutants with altered cell wall construction are also caffeine sensitive (267, 376, 389). In any case, Rlm1p appears to mainly affect the caffeine sensitivity of pathway mutants. Overexpression of *RLM1* suppresses the caffeine sensitivity of the *bck1*Δ MEKK mutant but not its temperature sensitivity (484). Cells with a *rlm1*Δ mutation are caffeine sensitive like *bck1*Δ but are not temperature sensitive (483). The temperature-sensitive phenotype of cell integrity pathway mutants may reflect decreased signaling to the SBF transcription factor, since *swi4* mutants are temperature sensitive (275, 348). To determine whether SBF and Rlm1p are the only targets of Slt2p, it would be interesting to determine whether the phenotype of a *swi4*Δ *rlm1*Δ double mutant copies that of a *slt2*Δ mutant.

Under conditions of mild heat stress, where the activity of the Slt2p kinase is increased by ~100-fold or more, there is either a 2- to 3-fold (484) or no (103) increase in Rlm1p-dependent expression of a reporter construct. It would thus appear that Rlm1p provides a strong, relatively constant induction to a subset of genes regulated by the MAPK cascade. A computer-assisted analysis of the genome for genes with a MEF2 consensus target sequence in their promoter uncovered several cell wall-related genes including those that encode the β-glucan synthesis regulator Hkr1p, the mannosyltransferase Ktr2p, and the flocculation protein Flo1p (103). However, whether the expression of these genes is cell integrity pathway-regulated has not yet been determined.

Two closely related proteins, Nhp6Ap and Nhp6Bp, also appear to act downstream of the MAPK cascade and may play a role in gene regulation (74). Nhp6p is a nuclear DNA binding protein related to mammalian HMG1 chromatin-associated proteins (216, 217). Cells lacking both *NHP6A* and *NHP6B* have a phenotype (74) similar to that of *slt2*Δ mutants (241), and a double mutation, *nhp6A*Δ *nhp6B*Δ, does not enhance the phenotype of *slt2*Δ mutants (74), indicating that Nhp6p acts in the same pathway as Slt2p. Overexpression of *NHP6A* or *NHP6B* suppresses a MEKK mutation (*bck1*Δ), but overexpression of *BCK1* does not suppress *nhp6A*Δ *nhp6B*Δ, demonstrating that Nhp6Ap and Nhp6Bp may act downstream of Slt2p (74). The way in which these two nuclear proteins help mediate cell integrity pathway responses is not yet known, but direct interactions with the MAPK Slt2p could not be detected (74).

Interaction with Other Pathways: Calcineurin and Calcium

In yeast, the phosphatase calcineurin plays a key role in supporting or mediating several important physiological processes. These include NaCl tolerance (43, 87, 128, 171, 289, 311, 329, 443, 488), mating (84, 85, 325, 493), intracellular calcium buffering (80, 81, 380, 455, 456), and construction of the cell wall (108, 115, 140, 301, 359, 443). Calcineurin, a Ca^{2+}- and calmodulin-regulated phosphoprotein phosphatase, is not required for normal cell growth of a wild-type strain (84, 85) but does appear to act together with the cell integrity pathway to perform a vital cell function (140, 330). A calcineurin null mutation is thus lethal in combination with *pkc1*Δ (140) or *slt2*Δ (140, 330), and the latter mutations confer increased sensitivity to the calcineurin inhibitors FK506 and cyclosporin A (140). Expression of a constitutively active form of calcineurin partially suppresses the temperature-sensitive cell lysis of *pkc1*Δ or *slt2*Δ strains (140). In addition, overexpression of Slt2p suppresses one phenotype of calcineurin mutants: the reduction in cell viability after pheromone treatment (330). Increasing the extracellular calcium chloride concentration partially rescues the temperature sensitivity of a *pkc1* mutant, whereas an equivalent concentration of $MgCl_2$ (100 mM) had little effect (247). It may be that this calcium-specific rescue is mediated through activation of calcineurin, although there are no further data to test this possibility.

The shared function of calcineurin and the cell integrity pathway appears to be related to cell wall construction and integrity. A major structural component of the cell wall, as stated above, is β-1,3-glucan, synthesized by the glucan synthases Fks1p and Fks2p. Single mutants with one of the glucan synthases removed are still viable, but removal of both is lethal (for reviews, see references 229 and 351). Cells with reduced calcineurin activity are sensitive to the loss of the glucan synthase gene *FKS1* (108, 115, 140, 359). Thus, in the absence of *FKS1*, *FKS2* becomes essential, and its expression is dependent on calcineurin (301). The calcineurin regulation of FKS2 expression is mediated through the transcription factor Crz1p-Tcn1p (297, 443). Mutations that inactivate *FKS1* are hence much more sensitive to the calcineurin inhibitors (108, 359) and are lethal in combination with null mutations in calcineurin (115, 140, 359) or Crz1p/Tcn1p (297). The reduced expression of *FKS1* in cell integrity pathway mutants (181) may thus account for their synthetic lethal phenotype with calcineurin mutants that have lower expression of *FKS2*. Other factors may contribute to the lethal phenotype of a mutant lacking both calcineurin and the MAPK cascade. For example, the cell integrity pathway also helps mediate the expression of *FKS2* (520).

There are three physiological situations in which increases in the calcium concentration appear to be temporally correlated with increased activity of the cell integrity pathway: during response to mating pheromone, at the G_1/S transition of the cell cycle, and after hypotonic stress. This correlation could reflect the coordinated activation of the cell integrity pathway and a calcium-activated pathway such one involving calcineurin and other calcium binding proteins. For example, pheromone activation of the cell integrity pathway MAPK Slt2p takes place ~15 to 30 min after pheromone addition (50, 118, 518). This response roughly correlates in time with a pheromone-induced increase in the concentration of cytosolic calcium (184, 328, 349). Cells deprived of calcium, like calcineurin mutants (84, 85), lose viability several hours after pheromone treatment (183, 184, 356). Cells lacking a functional cell integrity pathway also lose viability after pheromone treatment (118), suggesting that this pathway acts parallel to or is part of a calcium-induced response required for survival after pheromone treatment.

In another example, the cell integrity pathway is activated at the G_1/S transition (518) and helps induce increases in the expression of genes needed for cell wall construction in the bud (181). The intracellular calcium concentration has been suggested to increase during this time. For example, several proteins involved in the late G_1 and early S phase of the cell cycle have putative calcium binding sites: Cdc24p (317), calmodulin

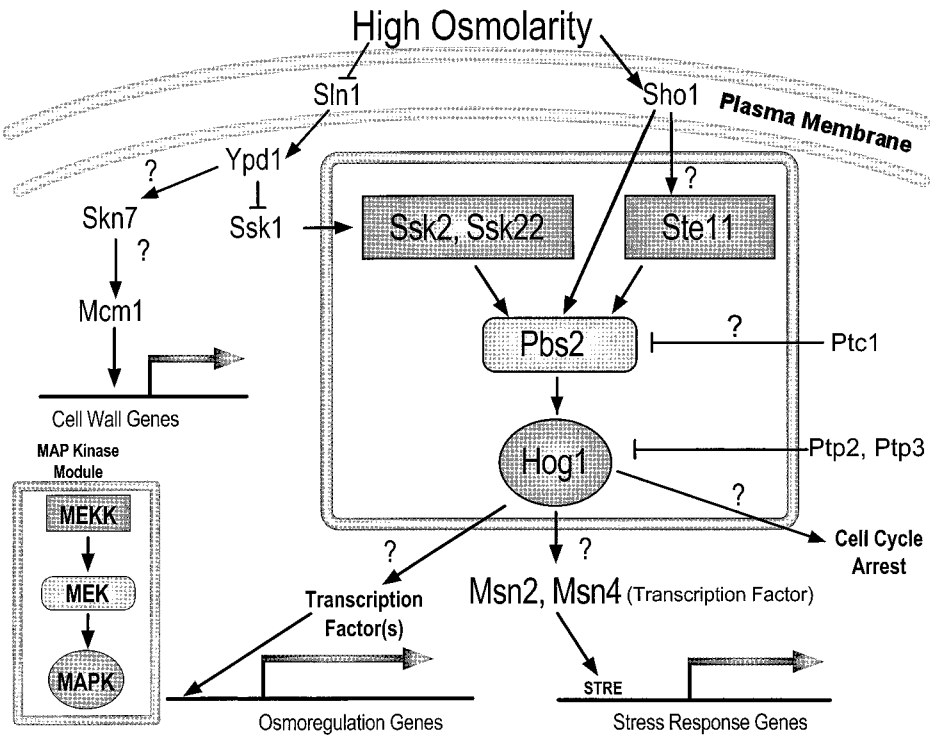

FIG. 5. HOG pathway of *S. cerevisiae*. The question marks show areas of uncertainty that require further investigation. Sln1p and Sho1p are assumed here to be present in the plasma membrane, but this has not yet been experimentally verified.

(90, 350), Cdc31p (24), Pin1p (454), and the glucan synthase regulator Hkr1p (504). Although calcium or manganese ions are needed to allow yeast to progress beyond the minibud stage of growth (265), it has not yet been demonstrated that the cytosolic calcium ion concentration increases in late G_1 or early S phase. Finally, another correlation is that hypotonic stress not only activates the cell integrity pathway (89, 201) but also induces an increase in the cytosolic calcium concentration (23, 26). The connections between Ca^{2+} signaling and cell integrity pathway activity could reflect an activation of the pathway by cytosolic calcium, regulation of calcium uptake by the pathway, or, as stated above, activation of parallel signaling pathways. Pkc1p has a putative calcium binding site (250), but its in vitro kinase activity is unaffected by calcium (12, 482). However, only a small fraction of the total cell Pkc1p can be tested for activity, making it difficult to interpret such negative results as representative of the total Pkc1p.

Interaction with the phosphatases Ppz1p and Ppz2p. Another set of signaling proteins that have a function related to that of the cell integrity pathway MAPK cascade are the closely related phosphatases Ppz1p and Ppz2p (240). Deletion of these phosphatases causes a temperature-sensitive cell lysis phenotype similar to that of mutants in the MAPK cascade (65, 240, 376). Overexpression of the phosphatases suppresses the phenotype of MAPK cascade mutants (240, 340). The phenotype of *ppz1*Δ *ppz2*Δ *slt2*Δ mutants is stronger than that of any single mutant (240), suggesting the phosphatases and the MAPK cascade are not on the same linear pathway but have some nonoverlapping functions. Consistent with this idea, MAPK pathway mutants are not Na^+ sensitive (375) whereas *ppz1*Δ or *ppz2*Δ cells show reduced NaCl-induced expression of the Na^+ transporter Ena1p and are Na^+ sensitive (375).

HOG PATHWAY

The internal osmolarity of a growing yeast cell is maintained higher than the external osmolarity. The resulting osmotic gradient across the plasma membrane brings in water for cell expansion and creates turgor. Increasing external osmolarity is a commonly encountered stress for a yeast cell in various natural environments such as a split-open grape drying under the sun, a petri plate left open in the incubator, or the start of a fermentation when sugar is added. Yeast cells are quite resistant to various types of stress including hypertonic stress (16, 401). This adaptability can be traced to stress-activated signaling pathways that sense the stress condition and activate expression of proteins that resist the toxic effects of the stress and promote survival and eventual cell growth under the new conditions. The HOG pathway and its MAPK cascade in budding yeast plays an important and somewhat specialized role in adapting to hyperosmotic stress. A similar pathway in fission yeast allows adaptation to not only hyperosomotic stress but also other stresses. We first discuss the HOG pathway in budding yeast and then move on to consider the related pathway in fission yeast, highlighting important differences and similarities.

The broad outline of the HOG pathway (Fig. 5) is fairly well described. The MAP kinase cascade consists of five protein kinases. Three MEKKs, Ssk2p, Ssk22p, (279), and Ste11p (378), activate a single downstream MEK, Pbs2p (38, 44, 281), that in turn activates a single MAP kinase, Hog1p (44, 281). The upstream part of the HOG pathway can be considered to have two incoming and partially redundant branches of signaling proteins that regulate the MEK Pbs2p (279). On one branch are the putative membrane protein Sho1p and the MEKK Ste11p. The other upstream branch of the HOG path-

way contains a three-component signaling protein complex composed of Sln1p, Ypd1p, and Ssk1p. These three proteins are structurally and functionally similar to regulatory proteins that form two-component or three-component phosphorelay systems in certain bacteria and also in plants and other eukaryotes (6, 13, 54, 261, 361, 412, 501). This three-component system regulates the function of the closely related and partially redundant MEKKs Ssk2p and Ssk22p (279, 281, 379). Increasing the osmolarity of the medium induces the expression of a large number of genes (33, 34, 172, 341, 342), a subset of which require the HOG pathway for induction (4, 5, 172, 343). The downstream substrates of the MAPK Hog1p that mediate this response are not yet known, although the transcription factors Msn2p and Msn4p (122) appear to be involved (148, 295).

The HOG pathway is required for part of the osmoregulatory response to an increase in osmolarity. Addition of various solutes, e.g., NaCl or sorbitol, at 0.1 to 1.5 M to a growing yeast culture induces an increase in the level of intracellular glycerol (35, 46, 336). The increase in the glycerol level is a result of two apparently independent processes. Under normal growth conditions, the glycerol permeability of the yeast plasma membrane is relatively high (46). However, hyperosmotic stress induces a rapid decrease in the glycerol permeability of the plasma membrane by inhibiting the function of Fps1p, a glycerol transporter and the major determinant of glycerol permeability of the plasma membrane (268, 450). In addition, the expression of enzymes needed for the synthesis of glycerol is induced (5, 36, 172, 341–343). High osmolarity induces the expression of *GPD1* and *HOR2*, encoding, respectively, the enzymes glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, which catalyze glycerol synthesis from dihydroxyacetone phosphate. The combined result of these responses is that yeast makes more and keeps more glycerol in response to hyperosmotic stress, raising the cytosolic glycerol concentration to molar levels (310, 391). This response can be considered osmoregulation because it works to raise internal osmolarity and restore the osmotic gradient.

In cells under hyperosmotic stress, the HOG pathway is not required for the Fps1p-mediated changes in glycerol permeability (176) but does appear to be involved in regulation of glycerol synthesis (44) through effects on expression of glycerol synthesis enzymes (5, 172, 342, 343). Strains with *HOG1* or *PBS2* deleted make less glycerol and do not grow at high osmolarity (44). Triple mutants lacking all three MEKKs are similarly osmosensitive, although any one MEKK (Ssk2p, Ssk22p, or Ste11p) can be sufficient to allow growth at high osmolarity (279, 378). Yeast has two genes encoding glycerol-3-phosphate dehydrogenase, *GPD1* and *GPD2*. *GPD1* is induced by hyperosmotic stress (5, 116, 172), while expression of the closely related *GPD2* is relatively unaffected by hyperosmotic stress (11, 116). Mutants defective for *GPD1* have an osmosensitive phenotype (5, 231), and the double mutant *gpd1*Δ *gpd2*Δ is even more osmosensitive (11). The severity of these phenotypes is roughly proportional to the reduction in the cellular glycerol level (11). Thus, the hyperosmotic stress-induced, HOG pathway-dependent synthesis of glycerol is required for growth in a high-osmolarity environment.

The osmosensitive phenotype of HOG pathway mutants probably reflects not just the reduced osmoregulation response but also a loss of other HOG pathway-dependent responses to hyperosmotic stress. The HOG pathway plays a role in mediating the hyperosmotic stress-induced expression of stress response genes (411), recovery of cell morphogenesis (45), and repression of the pheromone response pathway (158). Not only is the HOG pathway required for various responses to hyperosmotic stress, but also hyperosmotic stress activates the pathway, measured as an increase in tyrosine phosphorylation of the MAPK Hog1p (44, 281). The HOG pathway is activated by increasing the concentration of different solutes (e.g., NaCl, KCl, sorbitol, or glucose) (44, 89), showing that the activating stimulus is truly related to the osmotic change rather than an increase in concentration of a specific solute. How the MAPK cascade is activated by hyperosmotic stress stands out as a fascinating problem for which there are now several important molecular clues.

Regulation of a MAPK Cascade by a Three-Component System

As previously summarized, one of the upstream branches of the HOG pathway contains a phosphorelay system of three components, Sln1p, Ypd1p, and Ssk1p (379), that regulates the activity of two MEKKs Ssk2p and Ssk22p (279) (Fig. 6). These MEKKs in turn regulate the activity of the downstream MEK Pbs2p and MAPK Hog1p. A relatively linear signaling mechanism connects the activity of Sln1p, a putative osmosensor for the HOG pathway (281), to the activity of the MEKKs. Thus, Ssk1p is required for activation of the MEKK Ssk2p, a role that appears to involve its binding to the NH_2-terminal regulatory domain of Ssk2p (279, 377). In addition, it is the unphosphorylated form of Ssk1p that appears to activate the HOG pathway, as measured by increased phosphorylation of the MAPK Hog1p (279). These findings are particularly important because they show that one major class of signaling device, a three-component system, is physically and functionally connected to another major class of signaling device, a MAPK cascade.

Modulation of the activity of the MEKK regulator Ssk1p through changes in its phosphorylation state is mediated through the three-component system of Sln1p, Ypd1p, and Ssk1p functioning as a phosphorelay system. Sln1p appears to contain three functional domains (353, 379). Starting at its NH_2 terminus, the putative osmosensor domain of Sln1p has two predicted membrane-spanning hydrophobic segments with an intervening extracellular hydrophilic segment. Moving toward the COOH terminus, the putative sensor domain is followed by a histidine kinase domain and then a receiver domain containing a conserved aspartate residue. The postulated role of Sln1p as an osmosensor for the HOG pathway can be traced in part to the similarity between Sln1p and the *Escherichia coli* osmosensor EnvZ. The overall structure of EnvZ is similar to that of Sln1p, except that EnvZ lacks a COOH-terminal receiver domain (381).

The following mechanism for transferring phosphate from ATP to the MEKK regulator Ssk1p was revealed by an elegant series of genetic and biochemical experiments carried out by the Saito group (379). Figure 6 shows the route taken by that phosphate in the three-component system of the HOG pathway. First, Sln1p catalyzes the transfer of phosphate from ATP to a conserved histidine residue in its histidine kinase domain. In vitro reconstitution experiments with two mutant Sln1p proteins show that phosphate is transferred from the histidine of one Sln1p to the conserved aspartate residue in the COOH-terminal receiver domain of a second Sln1p. The intermolecular nature of this phosphotransfer reaction is confirmed by genetic analysis. Expression of a Sln1p with a defective or absent receiver domain or a defective or absent histidine kinase domain does not complement *sln1*Δ, but coexpression of both mutant proteins does complement *sln1*Δ (126, 353, 379). Whether phosphotransfer can occur between histidine kinase and receiver domains on the same Sln1p molecule is unknown.

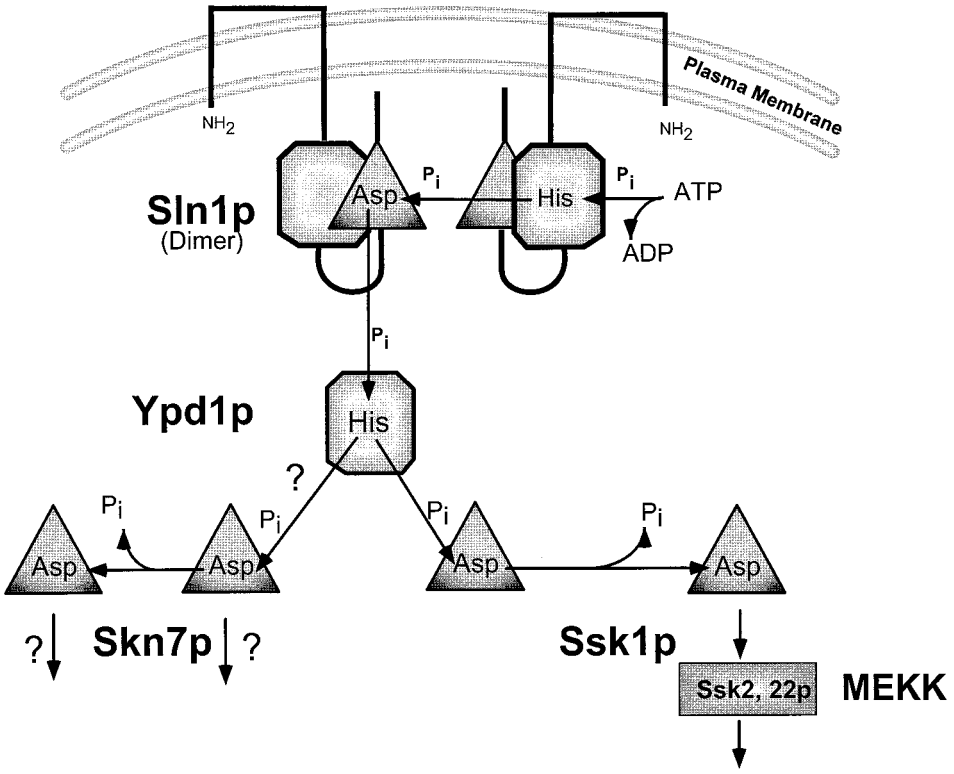

FIG. 6. Three-component system of the HOG pathway. Phosphate is transferred from ATP to a histidine residue on the histidine kinase domain (octagon) of Sln1p and from there to an aspartate residue on the receiver domain (triangle) of a separate molecule of Sln1p. Whether phosphate can be transferred from the histidine kinase domain to a receiver domain on the same Sln1p polypeptide chain as the former remains to be determined. Phosphate is then transferred from Sln1p to the histidine kinase Ypd1p, to either of two receiver domain proteins Ssk1p or Skn7p, and then to water. The phospho and dephospho forms of Skn7p have different functions. The dephospho form of Ssk1p is an activator of the HOG pathway MEKKs Ssk2p and Ssk22p; the phospho form of Ssk1p has no known function.

In vitro experiments (379) show that phosphate attached to the aspartate residue in the receiver domain of Sln1p is transferred to a histidine residue on Ypd1p and then from there to a conserved aspartate residue in the receiver domain of Ssk1p. These three proteins therefore form a phosphorelay system that moves phosphate from ATP to Sln1p-His to Sln1p-Asp to Ypd1p-His to Ssk1p-Asp. Mutation of any one of these four conserved amino acids in the three proteins completely blocks the negative regulation of the HOG pathway and results in hyperactivation of the MAPK cascade and subsequent toxicity (279, 281, 353, 379). Because the same phosphate is being transferred, there is no signal amplification in this process. The multistep nature of this phosphorelay system potentially allows regulation at any one of the steps. Moreover, observations of bacterial two-component signaling pathways (361, 447, 501) instruct us that both phosphorylation and dephosphorylation reactions are potential targets of regulation. Whether and how other signaling proteins in yeast regulate the activity of Sln1p-Ypd1p-Ssk1p is an important issue about which there is no current information.

From the model described above, Sln1p and Ypd1p are predicted to act as negative regulators of the HOG pathway MAPK cascade. Deletion of *SLN1* or *YPD1* is lethal unless HOG pathway signaling is blocked by deletion of *SSK1*, *SSK2*, *PBS2*, or *HOG1* (281, 379). Expression of Ssk1p with its receiver domain aspartate changed to a nonphosphorylatable asparagine is also toxic unless the downstream HOG pathway is blocked by mutation (279). Overexpression of the MEKKs Ssk2p or Ssk22p without their regulatory NH_2 terminus also induces HOG pathway hyperactivation and lethality unless the MEK Pbs2p or MAPK Hog1p is deleted. On the other hand, deletion of the upstream regulator *SSK1* cannot suppress the lethality of Ssk2pΔN overexpression (279). Negative regulation of MAPK cascades is therefore important in yeast because in three separate examples, i.e., the pheromone response pathway, the cell integrity pathway, and now the HOG pathway, hyperactivation is lethal. The HOG pathway is also negatively regulated by the action of the tyrosine-specific protein phosphatases Ptp2p and Ptp3p (153, 195, 352), which dephosphorylate the MAPK Hog1p (193, 500). Another negative regulator of the HOG pathway is the protein phosphatase Ptc1p (197, 280, 281), whose substrate is unknown. Removal or inactivation of these negative regulators causes reduced cell growth through hyperactivation of the HOG pathway. For example, *ptp2*Δ is lethal in combination with *ptc1*Δ (280). Overexpression of a downstream negative regulator Ptp2p can in turn suppress the lethal phenotype of a mutant lacking Sln1p, an upstream negative regulator (281).

Unphosphorylated Ssk1p activates the MAPK cascade (279), and Sln1p-Ypd1p relays phosphate from ATP to Ssk1p (379). The phosphorylation of Ssk1p is then predicted to inhibit its ability to activate the MEKKs Ssk2p and Ssk22p. This model makes several predictions. Increasing the osmolarity of the medium should inhibit the upstream phosphorelay proteins, leading to dephosphorylation of Sln1p, Ypd1p, and Ssk1p. Although the effect of hyperosmotic stress on the phosphorylation state of Sln1p or Ssk1p has not yet been reported, increasing the osmolarity of the medium induces a decrease in Ypd1p phosphorylation (379). This response provides stronger evidence that the upstream three-component is actually sens-

ing a change in osmolarity. One part of the model that seems incomplete is whether all the signaling in the system is mediated through modulation of phosphorylation reactions or whether phosphatase activity is being modulated by hyperosmotic stress. Another major unanswered question is how Sln1p senses a change in osmolarity. This is a tough question to answer. The function of the related osmosensor EnvZ has been studied for about 10 years (381), and its sensory mechanism is still under investigation (246, 360, 461–463, 506).

Activation of the MEKK Ssk2p by Ssk1p appears to be mediated by modulation of an Ssk2p autophosphorylation reaction similar to that used to induce MEKK activation in other MAPK cascades (92). Addition of unphosphorylated Ssk1p in vitro induces Ssk2p autophosphorylation. Autophosphorylation and activation of Ssk2p in the unstimulated cell appear to be blocked by an inhibitory domain at the NH_2-terminus (377), similar to that found in other MEKKs (314, 467). The receiver domain of Ssk1p binds close to this region of Ssk2p (377) and is therefore in a position to relieve the intramolecular inhibition of Ssk2p kinase activity. The binding of Ssk1p to Ssk2p is critical because increasing the external osmolarity induces autophosphorylation of wild-type Ssk2p but not of a Ssk2p mutant lacking the Ssk1p binding site (377).

Sln1p as a multifunctional protein. The function of Sln1p (and Ypd1p) in yeast appears to be more complex than just regulation of the HOG pathway MAPK cascade. Sln1p regulates the activity of Mcm1p-dependent genes, a function that is independent of the HOG pathway (126, 514). This role for Sln1p was uncovered in a screen for mutations that increase the expression of a Mcm1p dependent promoter-*lacZ* fusion (514). Mcm1p is a transcription factor that is essential for cells and that appears to form complexes with other transcription factors to regulate a variety of genes related to pheromone response, the cell cycle, and cell type specificity (111, 117, 180, 198, 225, 269, 304, 347). Expression of the *MCM1*-dependent reporter gene is increased by *nrp2* mutants, alleles of *SLN1*. Interestingly, deletion of *HOG1* (514) or even *SSK1* (126) has no effect on the *nrp2* phenotype. Furthermore, an increase in osmolarity of the medium, a HOG pathway-activating stimulus, has no effect on the expression of the *MCM1*-dependent reporter gene (514), although Mcm1p shows NaCl-induced changes in phosphorylation state (226). A *nrp2* mutation does not stimulate Hog1p phosphorylation (see below) (514). Hence, this function of Sln1p in regulating the activity of MCM1-dependent gene expression, as revealed by the *nrp2* alleles, does not appear to be mediated through activation of the HOG pathway. The genes regulated by the Sln1p-Mcm1p pathway are not fully characterized, although one such target appears to be *PIS1*, encoding phosphatidylinositol synthetase (8).

Two observations suggest that the *nrp2* mutants encode a hyperactivated form of Sln1p. First, *sln1*Δ cells, in contrast to the *nrp2* alleles of *SLN1*, show a decrease in expression of the *MCM1*-dependent reporter gene (514). Second, a *nrp2 sho1*Δ mutant shows an osmosensitivity similar to that of a *hog1*Δ strain and no detectable Hog1p phosphorylation in response to an increase in extracellular osmolarity (126). Deleting *SHO1* inactivates that upstream branch of the HOG pathway and makes cells dependent on the Sln1p-Ypd1p-Ssk1p-Ssk2/22p branch for activation of the HOG pathway (279). Inactivating Sln1p, the negative regulator of the HOG pathway, would activate the MAPK cascade. Instead, the *nrp2* mutation, in combination with *sho1*Δ, inhibits the pathway (126), suggesting that *nrp2* activates Sln1p function. One mechanism by which the *nrp2* mutations could activate Sln1p is by increasing the amount of phosphorylated Sln1p. The phosphorylation state of two-component regulators and thus their activity is regulated by the balance between autophosphorylation and autodephosphorylation (447). The observation that the *nrp* mutations are recessive to wild-type *SLN1* (514) suggests that the *nrp* mutation does not increase the autophosphorylation rate but instead inhibits the phosphatase activity of Sln1p, slowing its dephosphorylation.

The mechanism responsible for Sln1p activation of *MCM1*-regulated genes appears to involve the two-component proteins Skn7p and Ypd1p (96). Skn7p is a nonessential protein that contains a receiver domain with a conserved aspartate residue (48). In fact, Sln1p, Skn7p, and Ssk1p are the only proteins in yeast with a two-component receiver domain. Overexpression of *SKN7* activates expression of the *MCM1*-dependent reporter gene, while *skn7*Δ inhibits *MCM1*-dependent expression in strains expressing the *nrp2*-activated allele of Sln1p (96). Also implicated by deletion analysis as a mediator of the Sln1p regulation of the *MCM1* promoter is Ypd1p (96). Together, these data suggest a reciprocal control mechanism involving Sln1p and Ypd1p. In normal medium when the cell is growing and an osmotic gradient is present, Sln1p would be active in phosphorylating itself and relaying the phosphate through Ypd1p to Skn7p and Ssk1p. Phosphorylation of Skn7p would positively regulate the expression of *MCM1*-dependent genes, perhaps those involved in promoting cell cycle progression. Phosphorylation of Ssk1p would inactivate this MEKK activator, keeping the HOG pathway turned off. Increasing osmolarity or other types of stress would inactivate Sln1p, turning off the Skn7p functions related to growth and turning on Ssk1p functions such as HOG pathway activation that are related to stress resistance. This model may help explain why Skn7p appears to have different functions in mediating resistance to stress (219, 321) and in mediating the expression of G_1 cyclins and cell wall genes (47, 48). The conserved aspartate residue of the Skn7p receiver domain, the phosphoacceptor amino acid of Skn7p, is required for G_1 cyclin (321) and cell wall gene expression (47) but not for stress resistance (320).

SLN1 was originally identified in a screen for mutations that are lethal in combination with a *ubr1* mutation (353). Ubr1p is a protein that recognizes the NH_2 terminus of other proteins and marks them for degradation via a ubiquitin-mediated pathway (475). However, the connection between Sln1p and protein degradation pathways is still unclear and needs further investigation.

A Second Osmosensor and the Role of Pbs2p as a Scaffold Protein

A second upstream branch of the HOG pathway (Fig. 5) contains the putative membrane protein Sho1p and Ste11p, a MEKK found on other yeast MAPK cascades. As discussed above, the downstream protein kinases Pbs2p and Hog1p are regulated by a cascade of signaling proteins including a three-component system and two redundant MEKKs, Ssk2p and Ssk22p. However, single or double mutants with this part of the HOG pathway blocked upstream of Pbs2p are not osmosensitive like a *pbs2*Δ mutant (279, 281). Moreover, a *ssk2*Δ *ssk22*Δ mutant still shows induction of Pbs2p phosphorylation by hyperosmotic stress (378), showing that another MEKK can phosphorylate Pbs2p. To uncover genes in the missing upstream part of the HOG pathway, mutations that confer osmosensitivity only when combined with *ssk2*Δ *ssk22*Δ were isolated. This screen yielded mutations in *SHO1*, encoding a putative membrane protein with a COOH-terminal SH3 domain (279). The SH3 domain of Sho1p binds a proline-rich

segment in the NH_2 terminus of Pbs2p (279). A point mutation in this segment of Pbs2p blocks its interaction with Sho1p and has an osmosensitive phenotype in combination with *ssk2*Δ *ssk22*Δ but not by itself (279).

Increasing osmolarity induces the phosphorylation of a catalytically inactive form of Pbs2p, even in an *ssk2*Δ *ssk22*Δ strain (378). Based on its structure, Sho1p cannot be the missing protein kinase. Rather, Ste11p was identified as the third MEKK of the HOG pathway (378). An important observation is that a *ste11*Δ mutation confers osmosensitivity only when combined with *ssk2*Δ *ssk22*Δ (378); in fact, *ste11* was isolated by using the same screen that yielded *sho1*. Ste11p is required for in vivo hyperosmotic stress-induced phosphorylation of Pbs2p in a strain lacking the MEKKs Ssk2p and Ssk22p (378). In addition, Ste11p lacking its NH_2 terminus but retaining its kinase domain can phosphorylate Pbs2p in vitro (378).

The MEKK Ste11p is thus a signaling device that is used in three functionally distinct MAPK cascades in yeast (Fig. 1) (257, 378). Expression of a hyperactivated form of Ste11p (Ste11pΔN), made by deletion of its NH_2-terminal regulatory domain, is toxic to cells (51, 446). Deletion of the downstream pheromone response pathway and filamentation-invasion pathway MEK Ste7p or deletion of the downstream HOG pathway MEK Pbs2p does not suppress the lethality of Ste11pΔN expression. However, deletion of both MEKs, Ste7p and Pbs2p, completely suppresses the lethality of Ste11pΔN expression (378). The ability of a *ste11*Δ mutation to confer osmosensitivity to an *ssk2*Δ *ssk22*Δ strain is not the result of reduced signaling by the other Ste11p-dependent pathways. Deletion of *STE5* specifically blocks the pheromone response pathway; deletion of *STE20* or *STE7* blocks the pheromone response and filamentation-invasion pathways. These mutations, unlike *ste11*Δ, do not show synthetic osmosensitivity with *ssk2*Δ *ssk22*Δ (378).

The ability of Ste11p to function in separate pathways requires stable associations with pathway-specific proteins. Some of these interactions have been identified. For example, Ste11p interacts with the pheromone response pathway-specific scaffold protein Ste5p (61, 187, 287, 383). In the HOG pathway, the MEK Pbs2p serves as a scaffold protein, interacting with Ste11p (378) and Sho1p (279). A Ste11p binding scaffold protein specific for the filamentation-invasion pathway may exist but has not yet been identified. The pheromone response pathway and HOG pathway scaffold proteins may help bring together proteins that do not interact or interact only weakly but that are required for pathway signaling. For example, Ste11p shows no or very weak interaction with the MEK Ste7p in the two-hybrid system (61, 287, 383) and Ste5p binds both proteins (61, 187, 287, 383). Also, interaction between Sho1p and Ste11p is undetectable (378) and Pbs2p binds both proteins (279, 378).

The mechanism by which Ste11p is activated by hyperosmotic stress is unknown. For other MAPK cascades in yeast, there is an upstream activating protein kinase: Ste20p, the cell integrity pathway Pkc1p, or the spore wall assembly pathway-specific Sps1p. Ste20p (which acts on Ste11p in other pathways) and the Ste20p-related protein kinases Cla4p and Skm1p are not required for high-osmolarity growth of an *ssk2*Δ *ssk22*Δ mutant and are therefore not part of the Sho1p-Ste11p upstream arm of the HOG pathway. One of the important unsolved problems concerning the HOG pathway is therefore how the MEKK Ste11p is activated in response to hyperosmotic stress.

Mechanisms of Osmosensing

The mechanism by which the HOG pathway senses a change in extracellular osmolarity remains a major mystery. Defining which protein is an osmosensor, which physical parameter is being sensed, and how the sensor detects changes in that parameter are therefore important goals. Genetic and biochemical analysis of the upstream regulators of the MAPK cascade (279, 281, 378, 379) suggests that there are at least two different osmosensors, with Sln1p and Sho1p as the most likely candidates. One would expect that an osmosensor would span the plasma membrane, and Sln1p and Sho1p are each putative membrane proteins. However, the predicted structures of Sln1p and Sho1p are quite different from each other, suggesting a different role or function in sensing hyperosmotic stress. Measurements of the time and osmosis dependence of Hog1p phosphorylation in different mutant strains suggest that the two upstream branches of the HOG pathway may sense different physical parameters or react differently to the same parameter. Cells with *SHO1* and *SSK22* deleted show hyperosmotic stress-induced, Ssk2p-dependent changes in Hog1p phosphorylation (279). These changes are similar to those observed in wild-type cells (44, 279). In both cases, Hog1p phosphorylation appears within 1 min after addition of NaCl and is induced by 100 mM NaCl, with a maximum response at ~300 mM. In cells lacking *SSK2* and *SSK22*, hyperosmotic stress-induced, Sho1p- and Ste11p-dependent Hog1p phosphorylation cannot be detected until up to 300 mM NaCl is added and then only after 5 min since the addition of salt (279). The Ssk2p-dependent HOG pathway activation in *sho1*Δ *ssk22*Δ cells is thus faster and more sensitive than the Sho1p- and Ste11p-dependent response in *ssk2*Δ *ssk22*Δ cells.

Whether Sln1p and Sho1p are bona fide osmosensors remains uncertain. They are clearly required for hyperosmotic stress-induced phosphorylation of the HOG pathway MEK and MAPK. However, these two putative membrane proteins could function to correctly position other signaling proteins in the pathway to receive signal from the real osmosensor(s). To determine whether Sln1p or Sho1p are osmosensors, it will eventually be necessary to reconstitute the system from purified components and show that hyperosmotic stress regulation still occurs. One would predict that an osmosensor would alter its conformation or be chemically modified in response to osmotic stress. This has not yet been shown for Sln1p or Sho1p. However, the Ypd1p component of the Sln1p-Ypd1p-Ssk1p phosphorelay system in the HOG pathway does show, as predicted by the current model (379, 501), a decrease in phosphorylation after exposing cells to an increase in osmolarity (379). Turning to Sho1p, this putative membrane has an SH3 domain that interacts with Pbs2p (279). SH3 domains are involved in protein-protein interactions in yeast (29, 130, 298) and many other eukaryotes (67, 363). SH3 domains can regulate the catalytic activity of associated protein molecules (150, 168). Whether changes in osmolarity alter the interaction between the SH3 domain of Sho1p and Pbs2p has not yet been determined.

The physical parameter that is actually sensed by the HOG pathway has not yet been determined. This is a very difficult area in which to formulate specific molecular hypotheses. Increasing the osmolarity of the medium should reduce turgor pressure and decrease cell volume. Large-scale cell volume changes are not likely to be involved, because Hog1p phosphorylation reaches a maximum upon addition of NaCl to a final concentration of ~300 mM (44, 279), or approximately 500 mOsm. This change in osmolarity is too small to cause large changes in cell volume (15). The internal osmolality of

nongrowing yeast cells is estimated at ~600 mOsm (15), and growing cells have an even higher internal osmolarity (437). The osmolality of normal rich growth medium (YEPD) is approximately 250 mOsm. Thus, maximal activation of the HOG pathway appears to occur when the external osmolarity (~750 mOsm) is roughly similar to the internal osmolarity (>600 mOsm), suggesting the possibility that the HOG pathway is activated by a loss of turgor pressure.

How might a membrane protein such as Sln1p or Sho1p sense a change in turgor pressure? Turgor pressure is a stress that creates strain or tension in the cell surface. Thus, changes in tension in any of the components of the cell surface—the cell wall, plasma membrane, or plasma membrane-associated actin cytoskeleton—are potential signals. The cell wall is a complicated structure with many connections to underlying plasma membrane proteins. Changes in tension on these connections may cause conformational changes in cell wall-interacting plasma membrane proteins acting as osmosensors. Turgor may also create tension in the plasma membrane bilayer that can be a signal. Mechanosensitive ion channels in the yeast plasma membrane are activated by such tension (155). The cell integrity pathway is activated by chlorpromazine (201), a drug that intercalates into one leaflet of the lipid bilayer and creates tension (294). Perhaps the loss of this tension after an increase in external osmolarity may induce conformational changes in integral membrane proteins acting as osmosensors for the HOG pathway.

Cortical patches of actin filaments could be important for osmosensing. Electron micrographs of the yeast plasma membrane reveal many places where the membrane is invaginated (327). These membrane invaginations are wrapped with a helical bundle of actin filaments (327), forming a cortical actin patch (41). Changes in turgor could cause these invaginations to become deeper or shallower as the external osmolarity decreases or increases, respectively. The helical bundle of actin filaments may become correspondingly stretched or relaxed, leading to changes in conformation of actin-associated proteins acting as osmosensors. Whether Sln1p and/or Sho1p is present in these areas of the plasma membrane has not yet been tested.

Regulation of Gene Expression

An increase in external osmolarity is stressful to yeast and induces many physiological changes (176, 282). These include collapse of the osmotic gradient, temporary cessation of growth, loss of an organized actin cytoskeleton (63), temporary decrease in protein synthesis (282), induction of a subset of the heat shock proteins (33, 282, 411, 474), and increases in the concentration of intracellular solutes and macromolecules. If NaCl is used to increase the external osmolarity, there is additional stress due to the specific toxicity of this salt (418, 419). Increasing the osmolarity of the medium induces increased expression of many genes as measured by monitoring changes in mRNA or protein levels. This is a complex process in which the panoply of genes expressed depends on the solute used to increase the osmolarity and the extent of the increase in osmolarity. For a given osmotic stimulus, different genes show different time-dependent patterns of expression and extent of induction (33, 172). This suggests that the activities of many different *cis*- and *trans*-acting mechanisms are involved in determining the pattern of gene expression under conditions of hyperosmotic stress. Based on comparison of wild-type and HOG pathway mutants, the HOG pathway is required for the increased expression of many but not all of these genes (4, 5, 172, 343, 411). This analysis is not complete, but with new technology for examining the expression of all yeast genes now available (95, 232, 496), it should be completed soon.

Among the genes that show high-osmolarity-induced, *HOG1*-dependent expression, there appear to be at least three different regulatory mechanisms. One class of genes includes those that are induced by other stresses such as heat shock and that have a DNA sequence element called STRE in their promoter (123, 402, 431). STRE is different from the classical heat shock element (374). The catalase gene *CTT1* is a member of the STRE-regulated class of stress genes (411). Another class consists of genes whose induction does not involve STRE and appear to respond more specifically to hyperosmotic stress, for example, the glycerol-3-phosphate dehydrogenase gene, *GPD1* (116). Finally, the aldehyde dehydrogenase gene *ALD2* has no STREs and, in contrast to *GPD1*, is induced by a derepression mechanism (315). The transcription factors responsible for the induction of the last two classes of hyperosmotic stress-induced genes have not been identified. STRE-mediated gene expression appears to be mediated in part by Msn2p and Msn4p (148, 295, 407). Whether Hog1p directly interacts with Msn2p and Msn4p is not known. Cells lacking *MSN2* and *MSN4* show a reduction in the magnitude of hyperosmotic stress induction of several STRE-regulated genes including *CTT1*; however, the fold induction was relatively unchanged and some *PBS2*-dependent expression remained (295). Increasing osmolarity induces Msn2p to concentrate in the nucleus (148). Osmotic stress-induced nuclear translocation of Msn2p is unaffected by deletion of Hog1p. Instead, translocation of Msn2p to the nucleus appears to be negatively regulated by cyclic AMP and protein kinase A (148). Other proteins that appear to be required for hyperosmolarity-induced, *HOG1*- and STRE-dependent gene expression are Rox3p, a transcription factor, and Rts1p, a protein with homology to the regulatory subunit of phosphatase 2A (123). As with Msn2p and Msn4p, the mechanisms of interaction of Rox3p and Rts1p with the MAPK cascade need further investigation.

The regulation of gene expression in osmotically stressed or salt-stressed cells is clearly complex, with the HOG pathway cooperating with other pathways to regulate gene expression. For example, STRE-dependent gene expression is negatively regulated in many cases by the RAS-cyclic AMP-protein kinase A pathway (27, 39, 149, 286, 411, 474, 489, 492). This negative regulation appears to be mediated by cyclic AMP and protein kinase A-mediated inhibition of the STRE-binding transcription factors Msn2p and Msn4p (148, 436). Regulation of a STRE-*lacZ* reporter gene by the cyclic AMP pathway appears to be additive with that by the HOG pathway. For example, deletion of the Bcy1p regulatory subunit of protein kinase A causes constitutive activation of the protein kinase A (299) and reduces the expression of a STRE-*lacZ* reporter gene in response to hyperosmotic stress (411). However, *bcy1*Δ cells still show a relatively strong (10- to 15-fold) *HOG1*-dependent induction of the STRE-*lacZ* reporter gene by hyperosmotic stress (411). Increasing the osmolarity of the medium decreases cellular cyclic AMP levels (289), suggesting that a combination of a decrease in protein kinase A activity plus an increase in the MAPK Hog1p activity mediates the hyperosmotic stress induction of certain STRE-containing genes.

Another gene that shows combined regulation by the HOG pathway and a second pathway is *ENA1*, a gene encoding a P-type Na^+-ATPase responsible for Na^+ efflux from yeast (138, 161, 488). At low salt concentrations, induction of *ENA1* expression requires *HOG1*; at higher salt concentrations, the calcium-activated phosphatase calcineurin is required for *ENA1* induction (289).

A major unsolved question about the budding yeast HOG pathway is how Hog1p mediates the hyperosmotic stress-induced changes in gene expression. Hog1p (like the MAPK Slt2p [440]) is a strong transcriptional activator when fused to the Gal4p DNA binding domain (154). This activation may reflect an innate property of Hog1p or the recruitment of still-unidentified transcriptional activators to Hog1p. No substrates that act downstream of the MAPK Hog1p have so far been identified.

Regulation of the Pheromone Response Pathway

Each of the MAPK cascades in yeast responds to a specific signal, e.g., pheromone, an increase in osmolarity, nitrogen limitation, an increase in temperature, or induction of sporulation. One function of the HOG pathway MAPK cascade is to negatively regulate the pheromone response pathway and thereby inhibit activation by an inappropriate signal: hyperosmotic stress. The pheromone pathway MAPK Fus3p shows a small increase in tyrosine phosphorylation in response to high osmolarity, and deletion of *HOG1* or *PBS2* greatly enhances this response (158). Deletion of genes that code for members of the pheromone response pathway suppresses the osmosensitive growth phenotype of *hog1* and *pbs2* mutants (88). This suggests that an inappropriately activated pheromone pathway is deleterious for growth in high-osmolarity medium. Additional evidence for a role for HOG pathway regulation of the pheromone response pathway is the observation that cells lacking Hog1p or Pbs2p have an increased phosphorylation, transcription, and growth arrest response to exogenous pheromone addition (158, 445). The way in which the HOG pathway regulates the pheromone pathway and the mechanism of induction of pheromone response pathway by hyperosmotic stress are both unknown.

Regulation of Cell Growth

Transferring wild-type cells from normal growth medium to high-osmolarity medium induces a temporary growth arrest that is correlated with a loss in the normal distribution of the actin cytoskeleton (63). Actin filament patches, abundant in the growing bud (41), become more uniformly distributed between mother and daughter cells immediately after an increase in osmolarity and then later return to the original location upon resumption of growth (63). Mutants with mutations in the HOG pathway MAPK cascade show a similar initial response to hyperosmotic stress but later fail to restore actin filaments to the original bud. Instead, *hog1*Δ or *pbs2*Δ cells initiate the formation of a new bud, localize actin filaments to this new bud, and abandon the growth of the previous bud (45). This phenotype suggests that one function of the HOG pathway is to correctly position cell growth and division after hyperosmotic stress. The molecular mechanism by which Hog1p regulates this transition from arrested growth to resumption of growth is not known, but the HOG pathway appears to be required for sustaining cell cycle arrest in G_2 after an increase in osmolarity (7). Thus, the initiation of a new bud in the HOG pathway mutants may reflect cell cycle progression from S or G_2 to G_1 in a growth-arrested cell and formation of a new bud when growth resumes.

Turning off the MAPK Cascade

Addition of NaCl or other solutes to increase the extracellular osmolarity induces an increase in Hog1p tyrosine phosphorylation followed by a decrease back to near prestimulus levels (44, 193, 500). When cells contain a catalytically inactive Hog1p mutant in place of the wild-type protein, this mutant Hog1p does not show the later decrease in tyrosine phosphorylation after hyperosmotic stress (411, 500). This mutant is also osmosensitive (411). Thus, Hog1p-activated processes are required not only for adaptation to hyperosmotic stress but also for down-regulation of the HOG pathway. Constitutive activation of the pathway is lethal (279, 281, 500), even under hyperosmotic conditions (281), and so turning down the pathway activity is a vital function for yeast.

Hog1p could negatively regulate itself or the upstream part of the HOG pathway in several different ways. It could phosphorylate and inhibit upstream regulatory proteins. Alternatively, it could activate metabolic responses such as glycerol synthesis that restore turgor and turn off the osmosensors for the pathway. These two possibilities, involving a reduction in the rate of Hog1p phosphorylation, have not yet been investigated. However, one mechanism proposed (193, 500) for negative-feedback control of the HOG pathway is through Hog1p stimulation of the rate of Hog1p dephosphorylation by regulation of the expression or activity of Hog1p-specific phosphatases. This type of mechanism is known to mediate down-regulation of other MAPK cascades (70, 93, 152, 312). Mutational analysis (411) indicates that, like other MAPKs, Hog1p activity requires dual phosphorylation on a threonine residue and a tyrosine residue. Ptp2p and Ptp3p mediate the tyrosine dephosphorylation of Hog1p (193, 500); the threonine-specific phosphatase for Hog1p has not yet been identified. Thus, the late decrease in Hog1p phosphorylation after hyperosmotic stress is partially blocked by deletion of the tyrosine phosphatase Ptp2p and fully blocked by deletion of both Ptp2p and the Ptp2p-related phosphatase Ptp3p; a *ptp3*Δ mutation by itself has little effect on Hog1p phosphorylation (193, 500). Ptp2p thus appears to be the major tyrosine phosphatase for the HOG pathway MAPK, with Ptp3p playing a minor role; this situation is reversed in the pheromone response pathway, where Ptp3p is more important than Ptp2p in regulating Fus3p activity (519).

Whether Hog1p down-regulation is mediated through regulation of Ptp2p and Ptp3p expression or activity remains an open question. For example, hyperosmotic stress does induce *HOG1*-dependent increases in *PTP2* and *PTP3* mRNA levels (193), but the increases are relatively small and, in a separate study, barely detectable (500). Hog1p has been proposed to regulate Ptp2p and Ptp3p activity (500), but this possibility has not yet been experimentally investigated.

S. pombe Stress Response Pathway

The fission yeast *S. pombe* and the budding yeast *S. cerevisiae* have related signaling pathways, although the function and structure of these pathways have diverged in important ways. Fission yeast has a hyperosmotic stress-activated signaling pathway with a MAPK cascade closely related to that of the budding-yeast HOG pathway. However, the fission yeast pathway is much more of a general stress response pathway than the HOG pathway of budding yeast. The fission yeast pathway is activated not only by hyperosmotic stress (312, 426) but also by heat stress, oxidative stress, and nutrient limitation (93, 403, 426, 429). The stress-sensing pathway of fission yeast (Fig. 7) contains an upstream response regulator protein called Mcs4p (76, 319, 423, 429) that is structurally and functionally homologous to the HOG pathway protein Ssk1p (281). Mcs4p is required for stress-induced activation of a MAPK cascade composed of the MEKKs Wak1p (423) (also called Wis4p [403]) and Win1p (424) (unpublished results cited in reference 403), the MEK Wis1p (480), and the MAPK Stylp (312) (also

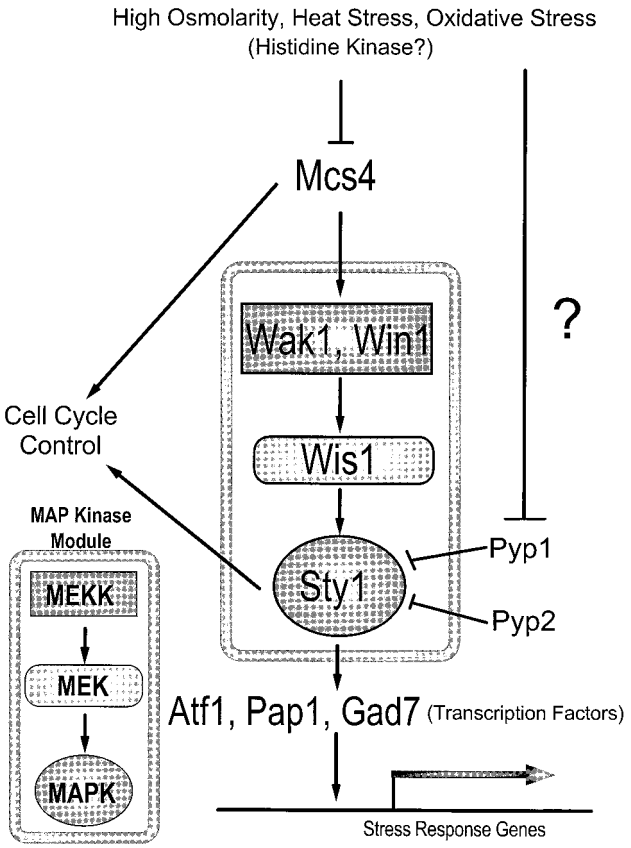

FIG. 7. Stress response pathway of *S. pombe*. Multiple stress signals activate the pathway, but the sensors for those signals have not yet been identified.

called Spc1p [426] or Phh1p [205]). This MAPK cascade stimulates the expression of numerous stress response genes (2, 137, 427, 464, 491). Like the budding-yeast HOG pathway (411), the fission yeast stress response pathway acts in opposition to a cyclic AMP pathway in regulating stress gene expression (444, 452). Transcriptional regulation by the MAPK cascade is mediated by the transcription factors Atf1p-Pcr1p (136, 427, 452, 485, 491), Gad7p-Pcr1p (203), and Pap1p (464). Protein phosphatases that negatively regulate this pathway include the tyrosine-specific phosphatases Pyp1p and Pyp2p (86, 160, 312, 313, 354, 426) (93) plus the phosphatases Ptc1p and Ptc3p (425, 428); the PP2C homologues Ptc1p and Ptc3p act downstream of the MAPK Sty1p (137).

Several features of the fission yeast pathway make it more similar to the mammalian stress-activated p38 pathway than to the budding yeast HOG pathway (490). Qualities shared by the fission yeast and mammalian pathways but not by the HOG pathway include activation by multiple stresses (93, 230, 429) and similar transcription factor targets for the MAPK (189, 427, 453, 464, 491). In addition, the mammalian and fission yeast pathways are both stimulated by the protein synthesis inhibitor anisomycin (230, 423) whereas the budding yeast HOG pathway is not (154).

The stress response pathway of fission yeast has several downstream branches. First, the upstream response regulator Mcs4p regulates cell cycle progression by both MAPK cascade-dependent and MAPK cascade-independent mechanisms (423). In addition, the MAPK cascade regulates cell cycle progression by a mechanism that is independent of its role in mediating activation of the transcription factor Atf1p (427, 491). The MAPK Sty1p regulates the transcription of many different genes with different transcription factors required for induction of specific genes. For example, Atf1p mediates the induction of osmotic stress genes and Pap1p mediates the expression of oxidative stress response genes (464).

The stress response pathway MAPK cascade plays an important role in the regulation of cell cycle progression. Mutations that promote the entry of fission yeast into mitosis produce shorter cells, whereas mutations that delay the entry into mitosis produce longer cells (273, 345). Hyperactivation of the stress response pathway by several means, including overexpression of the MEKK Wis1p, results in shorter cells (313, 480). Reduced activity of the pathway, e.g., by deleting protein kinases on the MAPK cascade, results in longer cells (312, 426, 480). Thus, the stress response pathway appears to act to promote entry into mitosis. Whether the regulation of the cell cycle by the stress response pathway is mediated by direct regulation of CDK activity or more indirect mechanisms remains unclear.

The apparent physiological function of the stress response pathway as a positive regulator of cell cycle progression predicts that stress would promote cell cycle progression. Indeed, nitrogen starvation, an activation signal for the stress response pathway (427), induces fission yeast to switch from mitotic division to sexual development (338, 508). This response to nitrogen starvation involves progression through mitosis to produce two G_1 cells that can either mate with a partner or enter stationary phase (72). The switch to mating competence is marked by the appearance of Ste11p, a transcription factor that induces genes required for pheromone production, cell fusion, etc. (338, 508). The stress response pathway plays a central role in this process. Not only does this pathway mediate cell cycle progression through mitosis to produce G_1 cells, but also it induces the Atf1p-dependent expression of the mating-specific transcription factor Ste11p (427, 452).

The stress response pathway of *S. pombe* not only is activated by stress but also is required for cell viability under the same conditions of stress that induce the activation of the pathway, for example, high osmolarity, heat shock, or the oxidant hydrogen peroxide (93, 205, 427). In contrast, heat shock or hydrogen peroxide does not appear to activate the *S. cerevisiae* HOG pathway (411), nor is the HOG pathway required for cell viability under these stress conditions (154). The mechanism by which a single pathway such as the fission yeast stress response pathway mediates responses to so many different stresses presents two problems. First, the pathway has to have sensors for each of these different stresses. These could be different sensor domain-histidine kinases for different signals or, instead, downstream proteins such as transcription factors, whose activity is directly regulated by a specific stress condition but not by others. For example, it has been suggested (464) that oxidative stress-induced activation of Pap1p might be mediated by changes in the oxidation state of certain cysteine residues in Pap1p, in a similar fashion to that observed for redox regulation of the bacterial transcription factor OxyR (94, 523). Additional inputs to the pathway could also confer stress sensitivity. For example, the Sty1p tyrosine phosphatases Pyp1p and Pyp2p appear to help mediate heat stress- and oxidative stress-induced changes in Sty1p phosphorylation, bypassing the need for Wis1p-mediated Sty1p phosphorylation (403). Hyperosmotic stress, on the other hand, requires Wis1p activity to induce Sty1p phosphorylation (403).

Second, the physiological requirements for adapting to one stress condition (e.g., hyperosmotic stress) are likely to be different from those for adapting to a different stress (e.g.,

oxidative stress). Therefore, the responses mediated by the stress response pathway are almost certainly different for different stresses. This has been shown for osmotic and oxidative stress responses, but the specificity of transcriptional responses to other stresses such heat stress has not yet been investigated. Another important factor in generating stress-specific responses could be another signaling pathway that is required in combination with the stress-activated Sty1p pathway to induce specific gene targets. One possibility for such a pathway is another MAPK cascade. Spm1p is an *S. pombe* MAPK that, like Sty1p, shows increased tyrosine phosphorylation in response to hyperosmotic stress and heat stress. Cells lacking Spm1p show morphogenetic defects and cell wall thickening under hyperosmotic stress or heat stress conditions (516). Cells lacking Mkh1p, a MEK, have a phenotype similar to that of an *spml* mutant, suggesting that Mkh1p acts in the same pathway (417). Mkh1p and Spm1p appear to act in a different pathway from Wis1p and Sty1p. The *spm1* phenotype is different from that of *sty1* mutants. In addition, Wis1p is required for stress induction of Sty1p but not Spm1p tyrosine phosphorylation (516). These results suggest that the Sty1p and Spm1p pathways work together to induce osmotic and heat stress responses, but whether these pathways act independently or are required together for activation of specific gene targets has not yet been determined.

SPORE WALL ASSEMBLY PATHWAY

When diploid yeast cells are exposed to a nitrogen-deficient medium that lacks a fermentable carbon source, they form spores that are resistant to a variety of stresses. Sporulation is a multistep process involving withdrawal from the normal cell cycle and sequential changes in gene expression to undergo meiosis I and II followed by assembly of a spore wall around each of four haploid nuclei (227). This developmental sequence of events is mediated by a number of different signaling proteins. A MAPK-containing pathway plays an important role in one part of this process, the assembly of the spore wall (220). Cells with a deletion in the MAPK gene *SMK1* fail to properly assemble spore walls and show reduced expression of late sporulation genes. It is not known whether the effects of *smk1*Δ on gene expression reflect a direct effect or a consequence of the failure to assemble a proper spore wall. *SMK1* is one of the middle sporulation genes (227) and is therefore expressed during the latter stages of meiosis and during the time when spore wall formation occurs. The Smk1p-containing spore wall assembly pathway is thus required for completion of a developmental pathway that has previously been induced.

Other signaling proteins that may be part of the Smk1p pathway are two protein kinases, Sps1p (131) and Cak1p (478). Sps1p is similar in sequence to members of the PAK subfamily of protein kinases. Like *SMK1*, *SPS1* is a middle sporulation gene and is required for proper spore wall assembly. Strains lacking *SPS1* or *SMK1* complete meiosis II but fail to properly assemble spore walls. Because three yeast MAPK cascades, the pheromone response pathway (236, 390), the filamentation/invasion pathway (257), and the cell integrity pathway (250), all have upstream activating protein kinases, perhaps Sps1p acts upstream of Smk1p on the same pathway (Fig. 8). However, in each of these other pathways, the upstream protein kinase activates a MEKK, but whether the Smk1p pathway contains a MEKK or MEK has not yet been determined.

Cak1p is an essential protein kinase that is required during vegetative growth for progression through the cell cycle (64, 120, 200, 415, 451, 459). The cell cycle function of *CAK1* is probably related to the ability of Cak1p to activate the cyclin-dependent protein kinase Cdc28p by phosphorylation (120, 200, 459). Important to the discussion here is that Cak1p also appears to regulate the Smk1p pathway. *CAK1* was isolated as a gene that, when overexpressed, suppresses the sporulation defect of a partial-loss-of-function *smk1* mutation but not that of *smk1*Δ (478). *CAK1* is expressed to a higher level during the same time that *SMK1* is expressed, and *cak1* mutants have a spore wall assembly defect similar to that of *smk1* cells (478). Whether Cak1p plays a supporting or instructive role on the Smk1p pathway has not yet been determined.

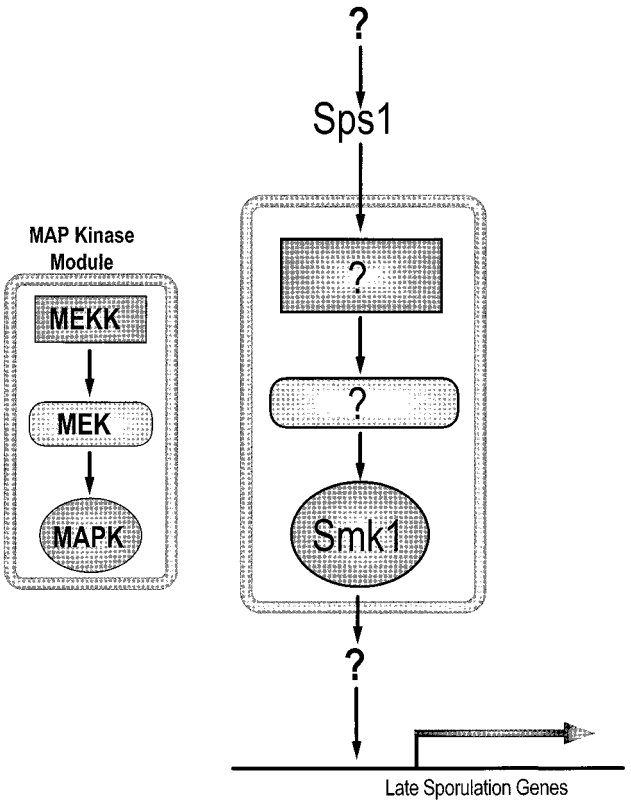

FIG. 8. Spore wall assembly pathway of *S. cerevisiae*. Whether signaling in the pathway is modulated solely by expression of pathway proteins or whether an upstream activating physiological stimulus exists has not yet been determined. A MEKK and MEK for this pathway have not been identified, and so it remains an open question whether this is a typical three-kinase MAPK cascade.

The mechanism by which Smk1p, Sps1p, and Cak1p coordinate the assembly of the spore wall is unknown. The phenotype of the *smk1*Δ or *sps1*Δ mutant is extremely interesting in its heterogeneity within a single cell. The spore wall has four different layers, consisting of two inner glucan layers, a chitin/chitosan layer, and an outer layer containing dityrosine-linked proteins. This multilayered structure is formed around each of the four nuclei within a single cell, the ascospore. A striking property of a *smk1*Δ or *sps1*Δ ascospore is that individual nuclei within the ascospore will have different spore wall defects, with cell wall layers missing, extranumerary, or improperly ordered. A different phenotype is observed when Smk1p activity is not eliminated but reduced (477). In strains expressing a temperature-sensitive Smk1p mutant or a smaller amount of wild type Smk1p, the spore wall appearance is uniform around each nucleus within an ascospore and arrested at a specific stage of spore wall formation. The stage at which spore wall formation is arrested correlates with the reduction in Smk1p expression of activity. This finding suggests the possi-

bility that ordered development of different spore wall layers is determined by time-dependent changes in the activity of the MAPK Smk1p (477). There is precedent for this notion: different levels of MAPK activation under regulation by fibroblast growth factor induce different types of mesoderm in *Xenopus* (471).

Thus, the Smk1p pathway, like the pheromone response pathway, has components that are expressed in only a certain cell type. It remains unclear whether the activity of the Smk1p pathway is merely proportional to the concentration of signaling protein(s) present in the cell during sporulation or whether the protein kinase activity of Smk1p (and Sps1p) is modulated by signals generated internally or externally during spore development. For example, it would be interesting to know whether there is any feedback from a spore wall layer to the Smk1p pathway. The way the Smk1p controls spore wall biogenesis is a fascinating biological problem that is relevant to many developmental processes. Another gap in our knowledge is whether there is a MEKK or MEK in the pathway or whether there is instead a new type of connection involved in regulating a MAPK, e.g., direct regulation by a PAK. It would also be interesting to determine whether Sps1p in fact is a PAK, p21-activated kinase.

CONCLUSIONS AND FUTURE DIRECTIONS

Given this overview of MAPK cascades in yeast, it is clear that several major questions remain unanswered for all of the pathways. We still do not have a clear picture of how the first protein kinase, the MEKK, of any of the yeast MAPK cascades is activated. MAPK is critical for ferrying signals to the nucleus and regulating transcription therein, but the dynamics of this process are only dimly understood. How is the MAPK released from cytoplasmic protein complexes, and how is the MAPK shuttled in and out of the nucleus? The phosphatases that dephosphorylate the protein kinases of the MAPK cascades and the downstream MAPK substrates are critical to shaping cellular responses and preventing the toxicity of pathway hyperactivation. The location of these phosphatases in cells and the question whether phosphatases are posttranslationally regulated are important issues that await further investigation. The phosphatases that act on yeast MEKKs or MEKs are yet unknown.

One important challenge is to determine how different pathways, activated under similar conditions of growth or stress, are used to coordinate complex morphogenetic functions such as cell fusion or cell restructuring from budding to forming mating projections. Another question is how in times of stress, the cell coordinates the function of its different pathways to achieve a balance between the competing needs for survival and cell growth. This coordination may involve cross talk between pathways acting at exactly the same time to uniquely induce or repress specific gene sets that require input from both pathways. The stress-activated MAPK pathways of budding and fission act in opposition to the cyclic AMP pathway (411, 452)—do these pathways act independently of each other, or is there cross talk at a higher level? Does the cell integrity pathway work together with Sps1p and the MAPK Smk1p to mediate spore wall assembly? Alternatively, does the coordination of pathways involve sequential activation of two pathways to allow one set of gene products to be made before another? One good example is that mating pheromone turns on first the pheromone response pathway followed by the cell integrity pathway (50, 118, 518). Other examples may exist; for example, severe hypotonic stress appears to activate Slt2p phosphorylation and then later Hog1p phosphorylation (88). The mechanism by which sequential activation (or deactivation) is achieved is not well understood. Finally, coordination of pathway functions may be used to create gain control. A small signal might activate one pathway, while a larger signal might activate that pathway and another. The investigation of the coordination of multiple pathways thus represents a major next step in understanding the control of cellular function in response to stress or growth signals.

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