Defects in Protein Glycosylation Cause *SHO1*-Dependent Activation of a *STE12* Signaling Pathway in Yeast

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ABSTRACT

In haploid *Saccharomyces cerevisiae*, mating occurs by activation of the pheromone response pathway. A genetic selection for mutants that activate this pathway uncovered a class of mutants defective in cell wall integrity. Partial loss-of-function alleles of *PGI1*, *PMI40*, *PSA1*, *DPM1*, *ALG1*, *MNN10*, *SPT14*, and *OCH1*, genes required for mannose utilization and protein glycosylation, activated a pheromone-response-pathway-dependent reporter (*FUS1*) in cells lacking a basal signal (*ste4*). Pathway activation was suppressed by the addition of mannose to hexose isomerase mutants *pgi1-101* and *pmi40-101*, which bypassed the requirement for mannose biosynthesis in these mutants. Pathway activation was also suppressed in *dpm1-101* mutants by plasmids that contained *RER2* or *PSA1*, which produce the substrates for Dpm1. Activation of *FUS1* transcription in the mannose utilization/protein glycosylation mutants required some but not all proteins from three different signaling pathways: the pheromone response, invasive growth, and HOG pathways. We specifically suggest that a Sho1 → Ste20/Ste50 → Ste11 → Ste7 → Kss1 → Ste12 pathway is responsible for activation of *FUS1* transcription in these mutants. Because loss of pheromone response pathway components leads to a synthetic growth defect in mannose utilization/protein glycosylation mutants, we suggest that the Sho1 → Ste12 pathway contributes to maintenance of cell wall integrity in vegetative cells.

VEGETATIVE yeast cells respond to environmental cues by activating signal transduction pathways that enable them to mount the appropriate physiological response. Among the cues that yeast responds to are mating pheromone, nutrients, osmolarity of the growth medium, and changes in turgor pressure at the plasma membrane. Each of these cues is dealt with by distinct signaling mechanisms to cause the appropriate response to a given stimulus.

Activation of the mating factor signal transduction pathway by binding of pheromone to its cognate receptor leads to a new pattern of gene transcription, to arrest of the cell division cycle in the G1 phase, and to reorientation of cell polarity toward the perceived mate (see reviews from Bardwell *et al.* 1994; Errede *et al.* 1995; Pringle *et al.* 1995; Herskowitz 1997; Leberer *et al.* 1997; Madden and Snyder 1998; Chant 1999). The signaling pathway is comprised of biochemical modules that are common to many transduction pathways in eukaryotic cells. At the head of the pathway is a serpentine seven-transmembrane receptor coupled to a heterotrimeric G protein. In ways that are incompletely understood, the activated G protein is coupled to a mitogen-activated protein (MAP) kinase cascade. This coupling involves Ste20, a protein kinase of the PAK family, and Ste5, a scaffolding protein for the MAP kinase cascade. The cascade itself is composed of Ste11 (an MAPKKK), Ste7 (an MAPKK), and Fus3 or Kss1 (two MAPKs). Either MAPK is capable of functioning in the pheromone pathway, but it is likely that Fus3 does so *in vivo* (Madhani and Fink 1998). Targets of the terminal MAPK include Ste12, a transcription factor required for transcription of pheromone-responsive genes, and Far1, a CDK inhibitor. These targets, therefore, constitute at least part of the mechanism whereby pheromone regulates transcription and cell cycle progression.

The third physiological effect of pheromone, reoriented cellular polarity, requires a different biochemical module, Cdc42, a p21 GTPase of the Ras superfamily. The membrane-tethered, activated G protein is thought to lead to localized activation of the guanine nucleotide exchange factor (GEF) for Cdc42 and, thereby, to localized activation of Cdc42 (Simon *et al.* 1995; Zhao *et al.* 1995; Nern and Arkowitz 1999). Once activated, Cdc42 can organize the actin cytoskeleton as it does in vegetative cells. Cdc42 may also activate Ste20 and influence signaling through the MAP kinase cascade (Stevenson *et al.* 1995; Pryciak and Huntress 1998; Johnson 1999).

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Although there is a fairly sophisticated understanding of signal transmission in the pheromone pathway, how signaling is regulated—for example, how the signal is attenuated so that the cell cycle can reinitiate once mating occurs—is poorly understood. Likewise, how the pheromone pathway may interface with other aspects of cell physiology is also poorly understood. These issues are brought into sharp focus by the realization that pheromone pathway components participate in other signal transduction pathways. For example, the haploid invasive growth phenotype, which is a response to nutrient limitation, requires Ste20, Ste11, Ste7, and Kss1 (Gimeno *et al.* 1992; Liu *et al.* 1993). Likewise, one branch of the HOG pathway, which is activated in response to osmotic stress, requires Ste20 and Ste11 (O'Rourke and Herskowitz 1998).

To learn more about regulation of the pheromone pathway and its possible interface with other pathways, we have sought mutants that showed increased expression of *FUS1*, a pheromone-responsive gene. A substantial number of these mutants had an ancillary slow-growth phenotype, and they proved to be defective at various steps in the pathway leading to the synthesis of mannosyl moieties or in the utilization of these moieties for protein glycosylation. Double-mutant studies argue that a distinct signaling pathway that begins with the membrane protein Sho1 for the HOG pathway and culminates at Ste12 from the pheromone response pathway is required for enhanced *FUS1* transcription. In addition, we found that mutations that debilitate protein glycosylation show synthetic interactions with mutations in pheromone pathway component structural genes, namely *STE20*, *STE11*, *STE7*, *KSS1*, and *STE12*. These results, which complement and extend those reported by Lee and Elion (1999), suggest that these components are part of a pathway that operates in vegetative cells to sense or respond to defects in the cell wall.

MATERIALS AND METHODS

Strains, media, and microbiological techniques: The yeast strains used in this work are listed in Table 1. The majority of the strains are isogenic with SY2002 (Stevenson *et al.* 1995), a derivative of Sc252 (Jim Hopper lab strain). The selection for His^+ mutants that activate a *FUS1-HIS3* reporter was performed in strains SY2428 (**a**) and SY2431 (α), derived from SY2002. These strains are *ste4*Δ *GAL-STE4 FUS1-HIS3 mfa2::FUS1-lacZ* and have complementary nutritional markers (*arg4* and *lys2*, respectively) to facilitate complementation analysis (Table 1). A strain containing the *alg1-1* allele was kindly provided by Phillip Robbins, and the strain containing the *spt14-2* allele was provided by Jan Fassler. Deletion of *STE* genes for epistasis analysis was performed using the one-step replacement plasmids *ste7::URA3*, *ste12::URA3*, *ste11::URA3*, *ste5::URA3*, *ste20::URA3*, *kss1::URA3*, *fus3-6::LEU2*, *ste12::LEU2*, and *ste4::LEU2*. The *mat***a***1*$^-$ strain was created by two-step replacement of construct YIp5-*MAT***a**Δ*Xho*1 to create a frameshift at the *HMR* locus followed by mating-type switching (using p*GAL-HO*) to obtain SY2428. The *STE11-4* allele was introduced into strains by transformation of the two-step replacement construct pSL1655. Other strains were constructed using one-step integration plasmids *bck1::URA3*, *slt2::URA3*, *rga1::URA3*, *pbs2::URA3*, *och1::LEU2*, *mnn10::LEU2*, and *gas1::URA3*. All strains that carried defined deletions were confirmed by Southern analysis or PCR Southern analysis, and by phenotypic analysis when possible. For construction of the *pmi40-101* and *pgi1-101* homozygous diploids, the **a** and α strains were transformed with complementary plasmids, and growth, mating, and selection for diploids were performed on 2% glucose with 10 mm mannose for *pmi40-101*, and on 2% mannose and 0.1% glucose for *pgi1-101*.

Yeast and bacterial strains were propagated using standard methods (Sherman *et al.* 1982). YPD and SD media have been described (Rose *et al.* 1990). Supplementation of media with 10 mm mannose (Sigma, St. Louis) was performed for analysis of strains carrying the *pmi40-101* allele. For strains that contained the *pgi1-101* allele, 2% mannose (or 2% fructose) and 0.1% glucose were used instead of 2% glucose. Yeast transformations were performed as described (Gietz *et al.* 1995). Calcofluor (white fluorescent brightener 28 Calcofluor white M2R; Sigma) was used at the concentrations described in the text. Bacterial transformations, bacterial DNA preparations, and plasmid constructions were performed by standard methods (Sambrook *et al.* 1989).

Plasmids: For genetic analysis of the mutants, deletion derivatives were produced by one- or two-step gene replacement using the following constructs. The *ste7::URA3* (pSL1077), *ste12::URA3* (pSL1311), *ste11::URA3* (pSL1094), and *STE11-4* (pSL1655) integration constructs were previously reported (Stevenson *et al.* 1992). The *mfa2-*Δ*1::FUS1-lacZ* (pDH17), *arg4* (YIp5*arg4-Eco*RV→*Xho*I), *rad16::pGAL::STE4* (pDH15), and *his3::FUS1-HIS3* (pDH106) plasmids have been described (Horecka and Sprague 1996). Constructs *ste5::URA3* (pSURE; J. Thorner), *ste20::URA3* (pEL45; Leberer *et al.* 1992), *kss1::URA3* (pGA1850; G. Ammerer), *fus3-6::LEU2* (pYEE98; Elion *et al.* 1990), *ste12::LEU2* (pSUL-16; Fields and Herskowitz 1987), and *ste4::LEU2* (p121; Whiteway *et al.* 1989) have also been described (Stevenson *et al.* 1992). Plasmids pRS313, 314, 315, and 316 have been described (Sikorski and Heiter 1989). The *bck1::URA3* and *slt2::URA3* plasmids were provided by D. Levin. The *rga1::URA3* and *pbs2::URA3* (Brewster *et al.* 1993) plasmids have been described previously (Stevenson *et al.* 1995). The *och1::LEU2* construct (Nakayama *et al.* 1992) and the *mnn10::LEU2* plasmids (Dean and Poster 1996) were described. Deletion of the *lys2* gene was performed with pCP7, using two-step integration, and YIp5-*MAT***a**Δ*Xho*I (Tatchell *et al.* 1981) was used to make strains *mat***a***1*$^-$. The *gas1::URA3* plasmid pSL1359 was made by digestion of the *URA3* gene from pSP65 with *Sal*I and insertion into the *GAS1* gene. Digestion with *Hin*dIII and *Eco*RI liberated the *gas1::URA3* fragment. For linkage analysis of *PGI1*, an *Eco*RI-*Sal*I fragment directly downstream of the *PGI*1 gene was cloned from a complementing YEp24 plasmid and introduced into the *Eco*RI and *Sal*I sites of pRS306 to create pPGI306. Digestion with *Cla*I was used to target integration directly downstream of *PGI1*. For linkage analysis of *PMI40*, a *Bam*HI-*Sal*I genomic fragment from a complementing YEp24 library plasmid was introduced into pRS306 by a directed sticky-end ligation to create pPMI306. The resultant plasmid was digested with *Cla*I to direct integration directly downstream of *PMI40*, creating a *URA3*-marked allele of *PMI40*. Plasmid pDPM306 was used to target integration of *URA3* directly adjacent to *DPM1*, creating a *URA3*-marked allele of *DPM1*.

Mutant isolation and analysis: Pilot experiments indicated that >30% of His^+ colonies that were isolated in a *ste4*Δ *FUS1-HIS3 his3* background also had a slow-growth defect. To gather a collection of independently isolated and spontaneous slow-

TABLE 1

Yeast strains

Strain	Relevant genotype	Source
SY2002[a]	*MAT***a** *his3::FUS1-HIS3 FUS1-lacZ*	Stevenson *et al.* (1995)
SY2003	*MAT*α *his3::FUS1-HIS3 FUS1-lacZ*	Stevenson *et al.* (1995)
SY2428	*MAT***a** *ste4*Δ *mat***a***1 arg4 GAL-STE4 his3::FUS1-HIS3 FUS1-lacZ*	This study
SY2431	*MAT*α *ste4*Δ *lys2 GAL-STE4 his3::FUS1-HIS3 FUS1-lacZ*	This study
SY3418	*MAT***a** *ste4*Δ *his3::FUS1-HIS3 FUS1-lacZ*	This study
SY3419	SY2431 *pgi1-101*	This study
SY3420	SY2431 *pmi40-101*	This study
SY3421	SY2431 *dpm1-101*	This study
SY3422	SY2431 *gas1-101*	This study
SY3423	SY2431 *pce1*Δ	This study
SY3424	SY2431 *rga1*Δ *pbs2*Δ	This study
SY3425	SY2002 *ste4*Δ[b]	This study
SY3426	SY2002 *ste4*Δ *och1*Δ	This study
SY3427	*ste4*Δ *FUS1-lacZ* in JF820	This study
SY3428	*ste4*Δ *spt14-2 FUS1-lacZ* in JF959	This study
SY3429	SY3418 *alg1-1*	This study
SY3430	SY3418 *mnn10*	This study
SY3431	SY2431 *gas1*Δ	This study
SY3432	SY2002/SY2003	This study
SY3433	SY2002/SY2003 *pmi40-101/pmi40-101*	This study
SY3434	SY2002/SY2003 *pgi1-101/pgi1-101*	This study
SY3435	SY2002 *pbs2*Δ	This study
SY3436	SY2002 *bck1*Δ	This study
SY3437	SY2002 *slt2*Δ	This study
SY3438	SY2002 *dpm1-101*[c]	This study
SY3439	SY2003 *dpm1-101*	This study
SY3440	SY2002 *dpm1-101 ste5*Δ	This study
SY3441	SY2002 *dpm1-101 ste50*Δ	This study
SY3442	SY2002 *dpm1-101 ste20*Δ	This study
SY3443	SY2002 *dpm1-101 ste11*Δ	This study
SY3444	SY2002 *dpm1-101 ste7*Δ	This study
SY3445	SY2002 *dpm1-101 fus3*Δ *kss1*Δ	This study
SY3446	SY2002 *dpm1-101 ste12*Δ	This study
SY3447	SY2431 *dpm1-101 ste5*Δ	This study
SY3448	SY2431 *dpm1-101 ste50*Δ	This study
SY3449	SY2431 *dpm1-101 ste20*Δ	This study
SY3450	SY2431 *dpm1-101 ste11*Δ	This study
SY3451	SY2431 *dpm1-101 ste7*Δ	This study
SY3452	SY2431 *dpm1-101 fus3*Δ	This study
SY3453	SY2431 *dpm1-101 kss1*Δ	This study
SY3454	SY2431 *dpm1-101 fus3*Δ *kss1*Δ	This study
SY3455	SY2431 *dpm1-101 ste12*Δ	This study
SY3456	SY2431 *dpm1-101 sho1*Δ	This study
SY3457	SY2431 *dpm1-101 ssk1*Δ	This study
SY3458	SY2431 *dpm1-101 tec1*Δ	This study
SY3459	SY2431 *dpm1-101 hsl7*Δ	This study
SY3460	SY2002 *dpm1-101 pbs2*Δ	This study
SY3461	SY2002 *dpm1-101 slt2*Δ	This study
SY3462	SY2002 *dpm1-101 bck1*Δ	This study
SY3463	SY2431 *dpm1-101 pbs2*Δ	This study
SY3464	SY2431 *dpm1-101 slt2*Δ	This study
SY3465	SY2431 *dpm1-101 bck1*Δ	This study
SY3466	SY2431 *gas1*Δ *dpm1-101*	This study

[a] SY2002, SY2003, SY2428, and SY2431 were derived from Sc252, a Jim Hopper lab strain; these strains are also *ade1 leu2 trp1 ura3 his3*.

[b] Wild-type strains containing *ste* gene disruptions have been made but are not listed here.

[c] Strains that contain the *pmi40-101* allele instead of the *dpm1-101* allele (for all *dpm1-101* strains) have also been made but are not listed here.

growth mutants, two single colonies, one *MAT*α (SY2431) the other *MAT***a** (SY2428), were inoculated into rich medium and grown in YPD for 16 hr at 30°. Approximately 5×10^8 cells were plated onto individual SD-HIS plates. His^+ colonies that had a slow-growth phenotype were picked from separate plates and characterized. Mating of *GAL-STE4 ste4*Δ strains for dominance/recessive and complementation tests was performed by first incubating cells for 5 hr in 2% galactose to induce the expression of *GAL-STE4*. Cells were then spotted on YPD plates in appropriate mating mixes for 18 hr and then spread onto selective plates. To facilitate the dominant/recessive test, SY2428 contained a mutation of the *MAT***a***1* gene, such that *mat***a***1*/*MAT*α diploids derived from this strain still expressed haploid-specific genes. A CEN-based plasmid containing the wild-type *MAT***a** gene was introduced into diploids to allow for sporulation. For a subset of the mutants, tetrad analysis was performed on synthetic medium due to the growth defect on YPD. For all of the mutants, segregation analysis demonstrated a 2 His^+:2 His^- ratio, indicating that the defect was due to a single gene. In addition, all the His^+ spores had a slow-growth defect, which showed that activation of the *FUS1* reporter cosegregated with slow growth. Complementation analysis was performed by mating the mutants in the **a** strain to the mutants in the α strain (as above) and assaying the diploids for growth on SD-HIS. Other tests for mating-specific functions were performed essentially as described (Sprague 1991), except that for *pgi1-101* and *pmi40-101* matings were performed in 2% mannose + 0.1% glucose and 2% glucose + 10 mm mannose, respectively. Linkage analysis was used to demonstrate that *PGI1*, *PMI40*, and *DPM1* were linked to the mutations represented by complementation groups 1, 2, and 3, respectively, in the following manner. Plasmids that contained genomic DNA directly adjacent to *PGI1*, *PMI40*, and *DPM1* were integrated into the genome of a wild-type strain. The resultant strains were mated to otherwise wild-type *pgi1-101*, *pmi40-101*, and *dpm1-101* mutants. Diploids were sporulated and tetrad analysis demonstrated that the Ura^+ phenotype segregated in repulsion to the temperature-sensitive phenotype for *pgi1-101*, *pmi40-101*, and *dpm1-101*. More than 20 tetrads containing four viable spores were assayed for each mutant.

Cloning the genes: Genes that complemented the slow-growth defect of the mutants were obtained by plasmid complementation using the library of Carlson and Botstein (1982). The DNA sequence of the complementing plasmids was obtained by the dideoxy chain termination method (Sanger *et al.* 1977) using double-stranded DNA templates and the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland). Subcloning of the library plasmids containing *PGI1*, *PMI40*, *DPM1*, and *GAS1* was used to show that these were the genes responsible for complementation of the slow-growth and *FUS1*-activation phenotypes. Additional *PMI40* clones were isolated using the λyes-galactose-inducible library (Elledge *et al.* 1991). Suppressors of the slow-growth defect of the *ste4*Δ *dpm1-101* strain were isolated using the λyes library and a CEN-based library (Rose *et al.* 1990; Rose 1991). The λyes library yielded *RER2*, and the CEN library yielded *PSA1*. Although the strains used in this study are isogenic to Sc252, we initially isolated *pgi1*, *pmi40*, and *gas1* alleles that activate *FUS1-HIS3* from the strain background 246-1-1 (provided by Kelly Tatchell; Stevenson *et al.* 1992), and in W303, suggesting that the relationship between protein glycosylation and *FUS1* activation is not a strain-dependent occurrence.

β-Galactosidase assays: For β-galactosidase assays, cells were prepared and assayed as described previously (Jarvis *et al.* 1988). Cells from an overnight culture were grown for 5 hr in YPD unless otherwise indicated. In the case of strains containing plasmids, cells were grown to saturation in selective medium before growth for 5 hr in YPD. The β-galactosidase activities reported are the average of three assays; standard deviation was <20%. For experiments involving tunicamycin, which is solubilized in DMSO, we confirmed that addition of DMSO alone had no effect on *FUS1-lacZ* activity.

Sequence analysis of *dpm1-101* and subcellular fractionation of the Dpm1 protein: The DNA sequence of the *dpm1-101* allele was determined by the sequencing of a single sample that contained a mix of five separate polymerase chain reactions (run for 20 cycles) of chromosomal DNA prepared from wild-type cells and from the *dpm1-101* mutant using primers that flank the *DPM1* gene. The *dpm1-101* allele had a single alteration from the wild-type sequence. Subcellular fractionation was performed by low- and high-speed centrifugation of cell lysates (Horazdovsky and Emr 1993). Protein fractions were precipitated with trichloracetic acid, run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with monoclonal antibodies specific for Dpm1. The blots were also probed for Pep12 and Vps10 as controls.

RESULTS

A subset of mutants that activate *FUS1* have an associated cell wall integrity defect: In a previous study, negative regulators of the pheromone response pathway were isolated by a selection for mutants that exhibited autonomous activation of the *FUS1* promoter (Stevenson *et al.* 1992, 1995). The selection was performed in a *ste4*Δ *his3::FUS1-HIS3* strain. As a consequence of the *ste4* mutation, the strain lacks basal signaling in the pheromone response pathway and the *FUS1* promoter is inactive. Hence, the strain is a histidine auxotroph. Selection of His^+ derivatives identified dominant and recessive mutations that activated pheromone response pathway signaling (Stevenson *et al.* 1992). The latter mutations are presumed to reveal genes specifying negative regulators of pheromone response pathway signaling. Indeed, one such mutation identified *RGA1*, which encodes a GAP for Cdc42, and suggested a link between Cdc42 and pheromone pathway components (Stevenson *et al.* 1995). The *ste4* mutation precluded organizing mutants into complementation groups. We therefore elected to carry out a large-scale selection in *ste4 his3 FUS1-HIS3* strains that also contain *GAL-STE4* (Table 1). Preliminary examination of 150 His^+ mutants showed that >30% had a slow-growth defect. Ten independently isolated His^+ colonies with a growth defect were characterized in greater detail. In addition to stimulation of the *FUS1-HIS3* reporter, the isolates showed enhanced expression of an integrated *FUS1-lacZ* reporter, as determined by β-galactosidase activity (Table 2). To assess whether the phenotype was dominant or recessive and due to a single mutation, the mutants were mated to complementary parent strains. All the diploids were His^-, indicating that the mutations were recessive. Tetrad analysis (of >20 tetrads per mutant) showed that for each mutant a defect in a single gene was responsible for *FUS1-HIS3* activation. Furthermore, for all the mutants the growth defect and *FUS1-HIS3* activation co-

TABLE 2

Quantitation of *FUS1* activation, Calcofluor sensitivity, and budding pattern in mutants

Relevant genotype or complementation group	Alleles	*FUS1-lacZ* (units)[a]	AT (mm)[b]	Calcofluor (μg/ml)[c]	Nonaxial budding (%)[d]
Group 1 (*ste4*Δ *pmi40*)	2	10	2	10	55
Group 2 (*ste4*Δ *pgi1*)	2	3	1	10	48
Group 3 (*ste4*Δ *dpm1*)	3	51	10	20	46
Group 4 (*ste4*Δ *gas1*)	1	1.5	1	20	45
Wild type	—	30	1	80	10
*ste4*Δ	—	<0.2	His^-	80	10
*ste4*Δ *pce1*	—	2	1	80	45
*ste4*Δ *rga1pbs2*	—	35	6	80	48
*ste4*Δ *STE11-4*	—	65	10	10	50

[a] β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of at least three independent determinations.

[b] The highest concentration of AT (aminotriazole) tested that allowed growth on synthetic plates lacking histidine.

[c] The highest concentration of Calcofluor tested that allowed growth on YPD plates.

[d] The percentage of nonaxial buds was determined for >200 cells in log phase. Cells were stained with Calcofluor white. The experiment was also repeated using wheat germ agglutinin; similar results were obtained.

segregated, implying that the same mutation was responsible for both phenotypes. Complementation analysis demonstrated that the mutations were not cell type dependent and that the mutants comprised at least four different complementation groups (Table 2).

We examined the slow-growth phenotype of the mutants in greater detail. *FAR1*-mediated G_1 arrest likely did not account for the growth defect of the mutants since other mutants that activated the pathway to the same or higher levels did not have a growth defect (Stevenson *et al.* 1992). The mutants grew more slowly at 37° than at 30°, and they also grew more slowly in YPD than in synthetic medium. Microscopic examination showed that the mutants had a common morphology: mutant cells were larger than wild-type cells, had multiple buds, and had a higher percentage of random or bipolar budding pattern (Table 2). A significant fraction of cells were observed to lyse when incubated at 37° or on YPD. We examined the possibility that cell lysis was due to a cell wall integrity defect by testing the mutants for sensitivity to the cell wall toxin Calcofluor. All the mutants of the slow-growth group were sensitive to Calcofluor (Ram *et al.* 1994; Table 2). However, not all His^+ mutants were sensitive to Calcofluor or exhibited the altered morphology (*e.g.*, *rga1*; Stevenson *et al.* 1995, and *pce1*; P. J. Cullen and G. F. Sprague, unpublished results; see Table 2), suggesting that the slow-growth mutants define a class of negative regulators of the pheromone response pathway. Strains containing the previously characterized *STE11-4* hyperactive allele (Stevenson *et al.* 1992) were also found to be sensitive to Calcofluor (Table 2). Because these mutants show increased expression of *FUS1*, a gene whose transcription is influenced by the pheromone response pathway but not other known pathways, we refer to the pathway activated in the mutants as the pheromone response pathway. However, this interpretation is revisited in the discussion.

Defects in mannose utilization and protein glycosylation cause activation of *FUS1* transcription: *PMI40:* To identify the genes responsible for the mutant phenotype, we selected for complementation of the growth defect after transformation with a plasmid library. A representative mutant from complementation group 1 was transformed with a YEP24-based yeast genomic library (Carlson and Botstein 1982), and transformants were selected for wild-type growth at 37°. Three independent isolates rescued the growth defect and also conferred a His^- phenotype to group 1 mutants (Figure 2). These plasmids contained overlapping genomic DNA fragments, each of which included the *PMI40* gene. Subsequent deletion analysis revealed that *PMI40* was indeed the complementing gene. To determine whether *PMI40* corresponds to group 1, *PMI40* DNA was cloned into pRS306 and the resulting construct was integrated at the *PMI40* locus by homologous recombination. In a genetic cross, the marked *PMI40* and a group 1 mutation segregated as alleles (no recombinants in >20 tetrads; see materials and methods), demonstrating that a defect in the *PMI40* gene was indeed responsible for *FUS1* reporter activation.

PMI40 encodes mannose-6-phosphate isomerase (Smith *et al.* 1992), which is required for synthesis of mannosyl moieties, important constituents of glycosylated proteins, and is therefore an essential gene (Figure 1). Addition of 10 mm mannose to growth media obviates the *PMI40* essential function (Smith *et al.* 1992). We therefore assessed the effect of mannose supplementation on the phenotypes of the *pmi40* allele isolated here, designated *pmi40-101*. *ste4*Δ *pmi40-101* cells were

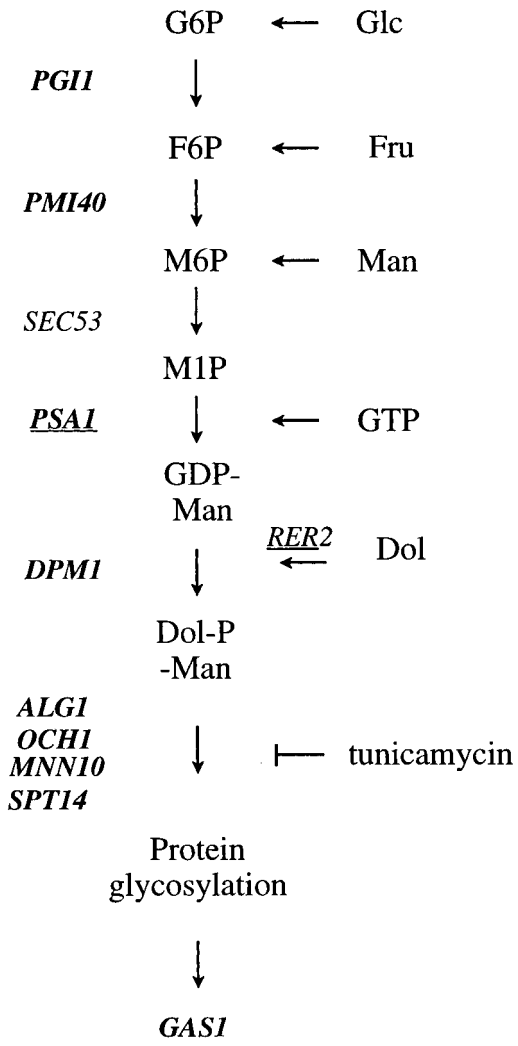

Figure 1.—Defects in mannose utilization and protein glycosylation lead to pheromone response pathway activation. Partial loss-of-function alleles of each gene shown in bold stimulated the *FUS1-lacZ* reporter in a *ste4*Δ background. Abbreviations are as follows: Glc, glucose; Fru, fructose; Man, mannose; P, phosphate; Dol, dolichol; GPI, glycophospholipid. Double arrows indicate that a number of enzymes are required for the given process. High-copy suppressors of the *dpm1-101* allele that also suppress pheromone response pathway activation are underlined. *GAS1* is the only target shown of the many targets of the N- and O-linked glycosylation and GPI anchor pathways.

able to grow at 37° on synthetic medium supplemented with 10 mm mannose, but they were histidine auxotrophs (Figure 2). That is, mannose supplementation rescued both the growth and pheromone pathway phenotypes of the mutants. To quantify the effect of mannose on *FUS1* transcription, we measured expression of *FUS1-lacZ*. The *ste4*Δ *pmi40-101* strain grown in the presence of 10 mm mannose had a β-galactosidase activity identical to that found in the *ste4*Δ parent strain (Table 3). Curiously, *ste4*Δ *pmi40-101* cells grown in YPD had greater β-galactosidase activity than the same cells grown in synthetic glucose medium (Table 3). Expression of *FUS1-lacZ* in wild-type or *ste4*Δ cells was not affected by mannose or by YPD. Together, these experiments imply that it is loss of Pmi40 enzymatic activity, not loss of an uncharacterized biochemical activity of the protein, that leads to activation of the *FUS1* reporters.

PGI1: Transformation of a group 2 mutant with the genomic library yielded independent complementing plasmids that contained the *PGI1* gene. Deletion mapping and subcloning verified that *PGI1* was the complementing gene, and integrative mapping showed that *PGI1* segregated as an allele of group 2 mutations. *PGI1* encodes glucose-6-phosphate isomerase, which converts glucose-6-phosphate to fructose-6-phosphate (Aguilera and Zimmermann 1986). Synthetic medium containing fructose as a carbon source (SF) supplemented with 5 mm glucose was previously shown to restore viability to *pgi1* mutants (Aguilera 1986). We showed that incubation in SF + 5 mm glucose medium suppressed both the growth defect and the His^+ phenotype of *ste4*Δ *pgi1-101* (not shown), suggesting that, as for Pmi40, it is loss of (or reduced) Pgi1 enzyme activity that leads to increased transcription from the *FUS1* promoter. Synthetic medium containing mannose as a carbon source and supplemented with 5 mm glucose also suppressed both phenotypes (see Figure 1). In addition, we observed an increase in *FUS1-lacZ* activity for *ste4*Δ *pgi1-101* mutants grown in YPD as compared to synthetic medium, as observed for *ste4*Δ *pmi40-101* cells (Table 3). The growth defects of mutants in the other complementation groups were rescued by neither SF + 5 mm glucose nor SD +10 mm mannose, suggesting that they did not owe their phenotype to diminished Pmi40 or Pgi1 activity. Thus, defects in 2-hexose isomerases that operate at the head of the mannose utilization pathway stimulated *FUS1* expression. Basal *FUS1-lacZ* activity in *pmi40* and *pgi1* strains containing an intact pheromone pathway (*STE4*) was more than twofold higher than in wild-type cells, which suggests that there is a physiological role for pheromone response pathway activation in mannose utilization mutants (see also the tunicamycin experiment below).

DPM1: Complementation of the slow-growth phenotype of a group 3 mutant yielded sets of plasmids from three distinct regions of the genome. One set of plasmids contained the *DPM1* gene, and tetrad analysis showed that complementation group 3 was linked to *DPM1* (see materials and methods). *DPM1* is required for synthesis of dolichol-P-mannose from dolichol and GTP-mannose (Orlean *et al.* 1988; Orlean 1990; Figure 1). A plasmid containing *DPM1* conferred wild-type growth and a His^- phenotype to all three mutants in this complementation group, and we therefore chose the strongest mutant in terms of *FUS1-lacZ* activation, *dpm1-101*, for further studies. As for the hexose isomerase mutants, *FUS1-lacZ* activity in *ste4*Δ *dpm1-101* was higher in YPD than in synthetic medium (Table 3). To

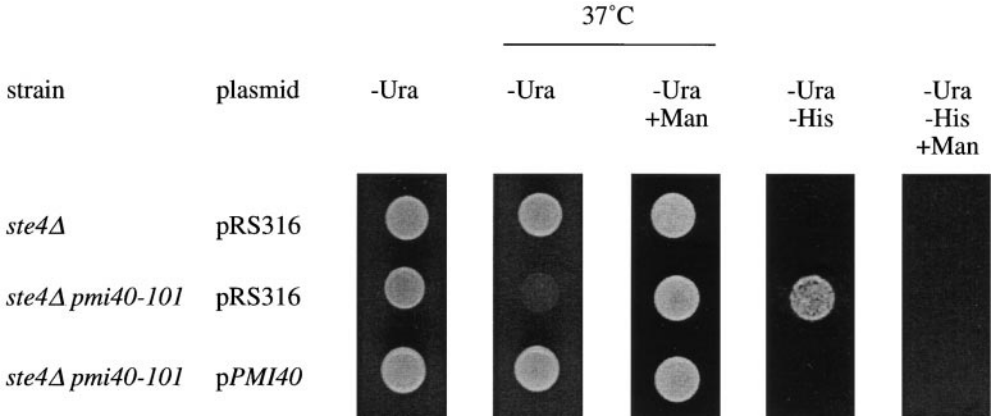

Figure 2.—The *pmi40-101* allele activates *FUS1-HIS3* in a *ste4*Δ background. Identical numbers of cells containing the indicated plasmids were spotted onto synthetic medium lacking uracil and with the following additional conditions: box 1, no modification; box 2, 37°; box 3, plus 10 mm mannose at 37°; box 4, minus histidine; box 5, minus histidine plus 10 mm mannose.

ascertain the nature of the defect of the Dpm1 protein, we examined *dpm1-101* in more detail. Western analysis using monoclonal antibodies to Dpm1 demonstrated that the amount and size of Dpm1 was identical in wild-type and *dpm1-101* strains (not shown). Subcellular fractionation profiles were also identical for strains containing wild-type and mutant Dpm1 proteins and were in accord with the reported endoplasmic reticulum localization of Dpm1 (not shown). The DNA sequence of *dpm1-101* contained a single nucleotide substitution (A to G) predicted to result in a glycine-to-serine substitution at amino acid 205. This position is adjacent to the predicted dolichol-binding site of Dpm1 (Albright *et al.* 1989; Orlean 1992). Because the *dpm1-101* allele was suppressed by overexpression of genes that provide either of the substrates for Dpm1 (see below), and because the level and localization of Dpm1G205S was identical to wild type, we suggest that Dpm1G205S has a defect in substrate binding or in the ligation of dolichol to mannose.

TABLE 3

Expression of *FUS1-lacZ* in mannose utilization mutants grown under different carbon sources

	FUS1-lacZ (units)[a]		
Genotype	SD + Man[b]	SD	YPD
Wild type	30	27	33
*ste4*Δ	<0.2	<0.2	<0.2
*ste4*Δ *pmi40-101*	<0.2	2.6	10
*ste4*Δ *pgi1-101*	<0.2	0.9	4
*ste4*Δ *dpm1-101*	9	10	51

[a] β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of at least three independent determinations.

[b] Except for *ste4*Δ*pgi1-101*, strains were grown in SD + 10 mm mannose for 16 hr. The cultures were then split into three aliquots, the cells were washed once by centrifugation, and then incubated in either SD + 10 mm mannose (SD + Man), SD only (SD), or rich medium (YPD) for 5 hr. *ste4*Δ*pgi1-101* cells were grown in 2% mannose plus 0.1% glucose for 16 hr, and for the SD + Man incubation, 2% mannose plus 0.1% glucose was used in place of 2% glucose and 10 mm mannose.

The screen for library plasmids that complemented the *dpm1-101* mutation yielded plasmids from two genomic regions distinct from the *DPM1* locus. One overlapping set of these suppressing plasmids contained the *PSA1* gene, which is required for the synthesis of GTP-mannose, one of the substrates for the Dpm1 enzyme (Hashimoto *et al.* 1997). The second set of plasmids contained *RER2*, whose product is important for dolichol synthesis (Sato *et al.* 1999), the other substrate for Dpm1. In addition to suppression of the growth defect of *ste4*Δ *dpm1-101*, the *RER2*- and *PSA1*-containing plasmids suppressed the His^+ and *FUS1-lacZ* activation phenotypes (Figure 3). We have also shown that a temperature-sensitive allele of the *PSA1* gene is capable of causing *FUS1-lacZ* activation in a *ste4*Δ strain at the nonpermissive temperature (not shown).

Other protein glycosylation steps: We hypothesized that mutants defective in subsequent steps of protein glycosylation might also show activation of the *FUS1* reporters, and we found support for this hypothesis in the following four experiments: (1) The *OCH1* gene, although not essential, encodes a protein that catalyzes mannose outer chain elongation of N-linked oligosaccharides. Deletion of *OCH1* in a *ste4*Δ background stimulated *FUS1-lacZ* expression by >15-fold (Table 4) and supported growth on SD-His medium. The *ste4*Δ *och1*Δ strain was sensitive to Calcofluor and grew more slowly on YPD than on synthetic medium. Microscopic examination of the *ste4*Δ *och1*Δ strain revealed the same morphology as that of *pgi1*, *pmi40*, and *dpm1* strains: cells were larger than wild type, and were observed to lyse in YPD or Calcofluor. (2) Loss of *ALG1* and *MNN10*, which are also required for N-linked glycosylation, activated *FUS1* transcription in *ste4* cells (Table 4). (3) The consequence of diminished GPI anchor addition was investigated by use of a temperature-sensitive allele of *SPT14*, which is required for the first step of GPI anchor addition (for review see Takeda and Kinoshita 1995). The *FUS1-lacZ* gene was integrated into a strain con-

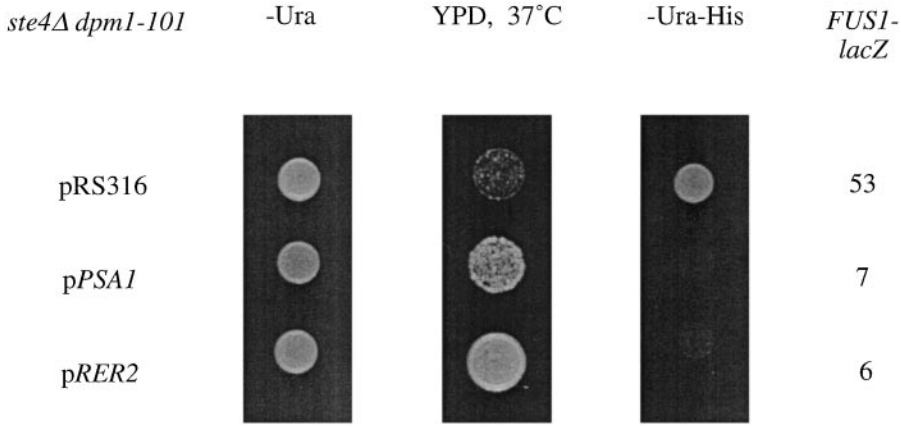

Figure 3.—Suppressors of the *ste4*Δ *dpm1-101* slow-growth phenotype also prevent *FUS1* transcription. Identical numbers of cells containing the indicated plasmids were spotted onto synthetic medium lacking uracil (left), YPD plates at 37° (center), and synthetic medium lacking both uracil and histidine (right). β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of three independent determinations.

taining a temperature-sensitive allele of *SPT14*, *spt14-2*, and the *STE4* gene was deleted. The *spt14-2* allele stimulated *FUS1-lacZ* by more than fourfold (even at 30°), and the strain was sensitive to Calcofluor (Table 4). (4) We hypothesized that inhibitors of protein glycosylation, such as the drug tunicamycin (Kuo and Lampen 1974), might also activate *FUS1*. Tunicamycin prevents N-linked glycosylation by acting as an inhibitor of the Alg7 protein (Lehle and Tanner 1976; Kukuruzinska and Lennon 1995). We found that addition of tunicamycin caused a >20-fold increase in *FUS1-lacZ* activity in *ste4*Δ cells (Table 4), comparable to loss of *OCH1*.

TABLE 4

Expression of *FUS1-lacZ* in mutants defective for protein glycosylation

Relevant genotype[a]	Calcofluor[b] (μg/ml)	*FUS1-lacZ*[c]
*ste4*Δ	80	0.37
*ste4*Δ *spt14-2*	10	1.6
*ste4*Δ *och1*Δ	10	5.7
*ste4*Δ *mnn10*Δ	10	1.4
*ste4*Δ *alg1-1*	10	1.5
*ste4*Δ	80	<0.2
*ste4*Δ + tunicamycin[d]	N/A	4.2
*ste4*Δ + tunicamycin (SD)[e]	N/A	0.6
Wild type	N/A	30
Wild type + tunicamycin	N/A	58

[a] Analysis of glycosylation mutants was performed in the W303-1A-derived *ste4*Δ cells containing an integrated *FUS1-lacZ* reporter. Cells were incubated at 37° for 5 hr for strains containing the temperature-sensitive alleles *alg1-1* and *spt14-2*. The tunicamycin experiments were performed in the SY2002 background.

[b] The highest concentration of Calcofluor tested that allowed growth on YPD plates.

[c] β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of at least three independent determinations.

[d] Tunicamycin was added at a concentration of 25 μg/ml to log-phase cultures; inductions were for 16 hr in YPD.

[e] Cells were induced in synthetic medium containing glucose.

Enhanced *FUS1-lacZ* expression was observed by the addition of tunicamycin to cells in YPD, reminiscent of the increased expression of *FUS1-lacZ* observed for *pgi1*, *pmi40*, and *dpm1* mutants. In wild-type cells grown on YPD medium, tunicamycin caused an approximately twofold increase in *FUS1-lacZ* expression. Therefore, *FUS1* transcription was activated by mutants defective in protein glycosylation and by tunicamycin, an inhibitor of protein glycosylation. Taken together, these results show that obstruction of the protein glycosylation machinery itself causes activation of the pheromone response pathway.

Loss of Gas1, a target of the protein glycosylation pathway, causes activation of *FUS1*: Isolation of plasmids that complement the slow-growth phenotype of a fourth mutant revealed that this group corresponds to *GAS1*. *GAS1* encodes an abundant and heavily N- and O-glycosylated protein that is the predominant GPI-anchored cell surface protein and is thus a major target of protein glycosylation (Popolo *et al.* 1997; Figure 1). Loss of *GAS1* results in the release of β-1,3-glucan into the medium and compromises the integrity of the cell wall, as shown in part by its synthetic lethality with loss of *PKC1* and *KRE6* (Popolo *et al.* 1997; Ram *et al.* 1998; Popolo and Vai 1999). As measured by degree of resistance to AT or by expression of *FUS1-lacZ*, the *gas1-101* allele caused only a modest activation of the *FUS1* reporters. *GAS1* is not an essential gene, and so we constructed a deletion allele to examine its effects. The phenotype of the *ste4*Δ *gas1*Δ strain was indistinguishable from that of the *ste4*Δ *gas1-101* strain (Table 5). The *ste4*Δ *gas1*Δ strain had a similar morphology to other protein glycosylation mutants that activate *FUS1*, and *gas1* was sensitive to Calcofluor. To address the possibility that loss of Gas1 function in some way precludes greater activation of the *FUS1* reporters, we measured β-galactosidase expression in a *ste4*Δ *gas1*Δ *dpm1-101* strain. As seen in Table 5, β-galactosidase activity in this strain was identical to that of a *ste4*Δ *dpm1-101* strain. One interpretation of these results is that the presumed global perturbation of cell wall structure caused by *dpm1-101* has a greater

TABLE 5

Loss of *GAS1* activates *FUS1* reporters in a *ste4*Δ strain

Relevant genotype	Calcofluor (μg/ml)[a]	AT resistance (mm)[b]	*FUS1-lacZ* expression[c]
Wild type	80	1	30
*ste4*Δ	80	His^-	<0.2
*ste4*Δ *gas1-101*	10	His^+	1.5
*ste4*Δ *gas1*Δ	10	His^+	1.5
*ste4*Δ *dpm1-101*	20	5	52
*ste4*Δ *gas1*Δ *dpm1-101*	10	5	55

[a] The highest concentration of Calcofluor tested that allowed growth on YPD plates.
[b] The highest concentration of AT (aminotriazole) tested that allowed growth on synthetic plates lacking histidine. His^+ refers to cells that grew on SD-His plates but not on SD-His + 1 mm AT.
[c] β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of at least three independent determinations.

effect on pathway signaling than does the loss of a single cell wall protein.

Components required for activation of *FUS1* transcription in protein glycosylation mutants: To determine the *STE* gene requirements for activation of the *FUS1* reporters in protein glycosylation mutants, *ste* mutations were introduced into the *dpm1-101* and *pmi40-101* strains. These strains were chosen because they show the highest *FUS1-lacZ* activity. Activation of *FUS1-lacZ* by *dpm1-101* was completely dependent upon *STE50*, *STE20*, the MAP kinase components *STE11*, *STE7*, and *KSS1*, and upon the *STE12* transcription factor (Table 6). Loss of *FUS3*, *STE4*, or *STE5* did not have an effect on *FUS1* transcription in *dpm1-101* cells, suggesting that proteins whose known functions are limited to the pheromone response pathway are not required for signaling to *FUS1* in the glycosylation mutants. Similar results were obtained for *pmi40-101* (not shown). Since *STE4* and *STE5* are haploid-specific genes and are not required for *FUS1* transcription in glycosylation mutants, we asked whether any haploid function was required by assessing *FUS1* expression in diploids defective for protein glycosylation. In diploids, the basal level of *FUS1* expression is dramatically reduced due to repression of haploid- and mating-specific genes (Trueheart and Fink 1989; McCaffrey *et al.* 1987). In the absence of mannose, the *FUS1-HIS3* and *FUS1-lacZ* reporters were activated in a homozygous *pmi40-101* diploid, as indicated by growth on media lacking histidine, as well as by a >15-fold increase in *FUS1-lacZ* activity. Neither reporter was activated in the homozygous *pmi40-101* diploid in medium supplemented with 10 mm mannose. Enhanced transcription of *FUS1* was also observed in homozygous *pgi1-101* diploid cells grown in the absence of mannose, whereas no significant increase in *FUS1* transcription was observed in wild-type diploids grown with or without mannose (data not shown).

The haploid invasive growth pathway requires the same subset of *STE* genes as are required for activated *FUS1* transcription in the protein glycosylation mutants (Roberts and Fink 1994; Tedford *et al.* 1997). We therefore tested the role of genes thought to be involved in invasive growth, not mating. Loss of Tec1, a transcription factor required for invasive growth but not mating or *FUS1* transcription (Madhani *et al.* 1997), had no effect on *FUS1-lacZ* expression in either *dpm1* or *pmi40* cells (Table 6; data not shown). Likewise, loss of Hsl7,

TABLE 6

Effect of *ste* mutations on *FUS1-lacZ* expression in *dpm1-101* mutants

Genotype	*FUS1-lacZ* expression[a]	AT resistance (mm)[b]
Wild type	30	1
*ste4*Δ[c]	<0.2	His^-
dpm1-101	73	5
*dpm1-101 ste4*Δ	50	2
*dpm1-101 ste5*Δ	75	5
*dpm1-101 ste20*Δ	<0.2	His^-
*dpm1-101 ste50*Δ	<0.2	His^-
*dpm1-101 ste11*Δ	<0.2	His^-
*dpm1-101 ste7*Δ	<0.2	His^-
*dpm1-101 ste4*Δ *fus3*Δ	55	5
*dpm1-101 ste4*Δ *kss1*Δ	4	His^+
*dpm1-101 ste4*Δ *fus3*Δ *kss1*Δ	<0.2	His^-
*dpm1-101 ste12*Δ	<0.2	His^-
*dpm1-101 ste4*Δ *tec1*Δ	53	2
*dpm1-101 ste4*Δ *hsl7*Δ	48	2
*dpm1-101 ste4*Δ *sho1*Δ	<0.5	His^-
*dpm1-101 ste4*Δ *ssk1*Δ	52	2

[a] β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of at least three independent determinations.
[b] The highest concentration of aminotriazole (AT) tested that allowed growth on synthetic medium lacking histidine. His^+ refers to growth on SD-His plates but not on SD-His + 1 mm AT.
[c] Deletion of other *STE* genes in a wild-type cell gave β-galactosidase activity equivalent to that seen in *ste4*Δ (<0.2 units).

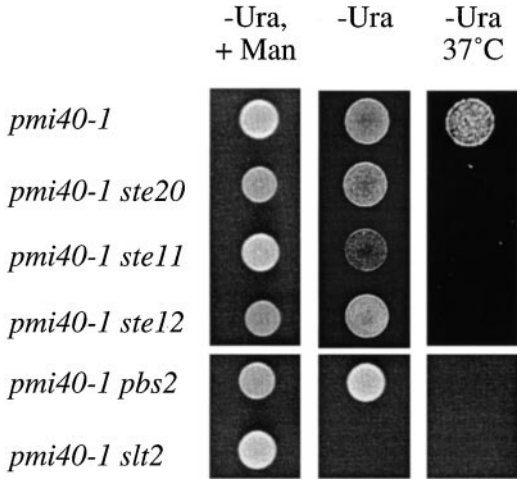

Figure 4.—Synthetic interactions of *pmi40*. Equal concentrations of cells were spotted onto synthetic media containing 2% glucose + 10 mm mannose at 30° (left), 2% glucose at 30° (middle), or 2% glucose at 37° (right).

TABLE 7

The PKC pathway mediates *FUS1-lacZ* expression in *dpm1-101* cells

Genotype	*FUS1-lacZ* expression[a]	AT resistance (mm)[b]
Wild type	30	1
bck1	31	1
slt2	27	1
ste4	<0.2	His$^-$
ste4 bck1	<0.2	His$^-$
ste4slt2	<0.2	His$^-$
dpm1-101	73	5
dpm1-101 bck1	30	5
dpm1-101 slt2	34	5
dpm1-101 ste4	55	1
dpm1-101 ste4 bck1	16	1
dpm1-101 ste4 slt2	3	His$^-$
Wild type (pRS314)	28	1
Wild type (p*BCK1*-20)	108	10

[a] β-Galactosidase activity was determined as described in materials and methods. The reported values are the average of at least three independent determinations.

[b] The highest concentration of aminotriazole (AT) tested that allowed growth on synthetic medium lacking histidine.

a negative regulator of Ste20 function in invasive growth (Fujita *et al.* 1999), had no effect on *FUS1-lacZ* expression in *dpm1-101* strains (Table 6), which further supports the fact that components specific for the invasive growth pathway are not required for signaling to *FUS1*.

In some HOG pathway mutants, namely *pbs2* and *hog1* mutants, Ste20-dependent cross talk to the pheromone response pathway is observed (O'Rourke and Herskowitz 1998; Sprague 1998). We therefore asked whether loss of either the Ssk1 (Maeda *et al.* 1994) or the Sho1 (Maeda *et al.* 1995; Posas and Saito 1997) branch of the HOG pathway affected activation of *FUS1* reporters. We found that *SHO1* was absolutely required for signaling to *FUS1* in *dpm1-101 ste4*Δ cells (Table 6) and in *pmi40-101 ste4*Δ cells (not shown). Loss of *SHO1* did not affect signaling in wild-type or *ste4*Δ cells, consistent with previously published evidence that *SHO1* is not required for *FUS1-lacZ* transcription or for mating (data not shown; O'Rourke and Herskowitz 1998). Deletion of *SSK1* had no effect on signaling in *ste4*Δ *dpm1-101* (Table 6) or *ste4*Δ *pmi40-101* cells (not shown).

Loss of *STE* pathway components exacerbates the growth defect of protein glycosylation mutants: In the course of the epistasis analysis, we noticed that deletion of *STE20*, *STE11*, or *STE12* exacerbated the growth defect of *pmi40-101* mutants (Figure 4), whereas deletion of *FUS3*, *STE4*, or *STE5* (which are not required for *FUS1* signaling by *pmi40-101*) had no effect on cell growth (not shown). The growth defect was completely rescued by supplementation with 10 mm mannose, which bypasses the requirement for Pmi40. Loss of *STE11* in *pmi40-101* cells caused a slightly more severe growth defect than loss of the other *STE* genes, suggesting that *STE11* has a function in *pmi40-101* cells in addition to its participation in the pheromone and invasive growth pathways, perhaps as a participant in the HOG pathway (see below). Loss of *STE* gene function also conferred a synthetic growth defect in the *dpm1-101* background. The synthetic defect was less severe than in the *pmi40-101* background, perhaps because *pmi40-101* alone has a more severe growth defect than does *dpm1-101* (Table 2). We also found that the previously characterized hyperactive *STE11-4* allele caused Calcofluor sensitivity and lysis (Table 2). Epistasis analysis showed that *STE7*, *FUS3*/*KSS1*, and *STE12* (but not *STE4* and *STE5*) were required to mediate Calcofluor sensitivity in a *STE11-4* strain (not shown). These sets of results imply that pheromone response pathway components participate in maintaining cell wall integrity in vegetative cells.

Intact protein kinase C and HOG pathways are essential in protein glycosylation mutants: Given that the protein glycosylation mutants exhibit a propensity to lyse and given that Ste20, Ste50, and Ste11 have a known role in the HOG pathway as well as in the pheromone and invasive growth pathways, we postulated that glycosylation pathway mutations would show synthetic interactions with the HOG pathway mutations. Indeed, loss of *PBS2* caused a growth defect in *pmi40-101* cells (Figure 4). The growth defect of *pbs2 pmi40-101* cells was comparable to *ste20 pmi40-101* or *ste12 pmi40-101* cells, but less severe than that of *ste11 pmi40-101* cells. Together, these results imply that components from both the pheromone/invasive growth pathway (Ste7, Kss1, and Ste12) and the HOG pathway (Pbs2) are required to maintain viability in protein glycosylation mutants.

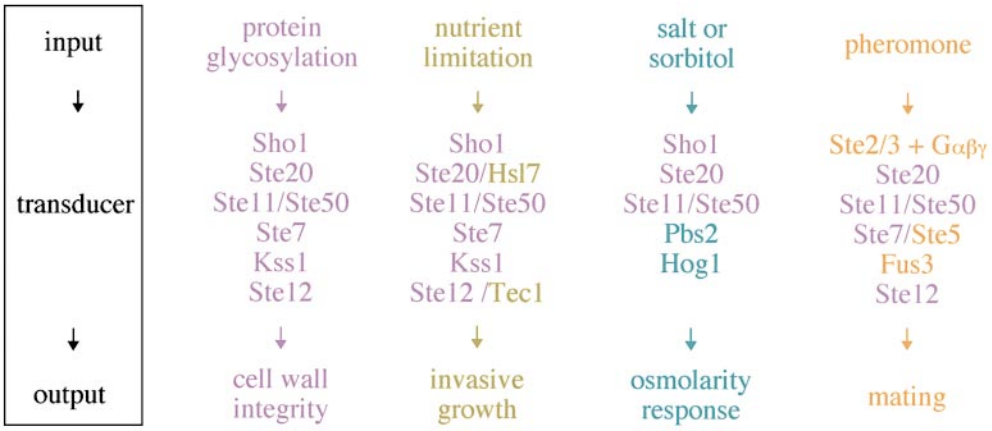

Figure 5.—A Sho1 → Ste12 signaling pathway. The inputs, signal transducers, and outputs of each Ste11-dependent process are shown. Orange represents components specific for the pheromone pathway, green represents components for the HOG pathway, and dark yellow depicts components for the invasive growth pathway. Proteins in purple are used in each of the pathways.

We also hypothesized that the protein kinase C (PKC) pathway, which controls cell wall integrity and can be modulated by the pheromone response pathway (Yashar *et al.* 1995; Buehrer and Errede 1997), might have a positive function in mutants compromised for protein glycosylation. As in the pheromone and HOG signaling pathways, a MAP kinase cascade is required for PKC-dependent signaling (Lee and Levin 1992; Irie *et al.* 1993; Mazzoni *et al.* 1993). Loss of the MAPKKK Bck1 or of the MAPK Slt2 was lethal in a *pmi40* strain (Figure 4; data not shown). Thus, loss of PKC pathway components caused a more severe growth defect than loss of the HOG pathway components in *pmi40-101* cells. Deletion of either Bck1 or Slt2 exacerbated the slow-growth phenotype of *dpm1-101* cells. However, the double mutants were viable, which allowed *FUS1-lacZ* analysis in these strains. Loss of *BCK1* or *SLT2* decreased *FUS1-lacZ* expression in the *dpm1-101 ste4*Δ background by >10-fold, and in *dpm1-101* by >2-fold (Table 7). This latter result suggests that the PKC pathway may influence signaling in the pheromone response pathway in wild-type cells. As another means to test this possibility, we used a hyperactive allele of *BCK1*, *BCK1-20* (Lee and Levin 1992). Plasmid-borne *BCK1-20* stimulated the *FUS1-lacZ* reporter by more than threefold in the wild-type strain (Table 7). In addition, *BCK1-20* caused enhanced growth of wild-type cells on medium lacking histidine, presumably due to enhanced expression of the *FUS1-HIS3* reporter. Epistasis analysis showed Ste11, Ste7, and Ste12 were required for the *BCK1-20* effect (not shown). Thus, the PKC pathway is required for viability, and may contribute to *FUS1* expression, in protein glycosylation mutants.

DISCUSSION

Defects in protein glycosylation activate *FUS1* transcription: We have presented several lines of evidence that defects in protein glycosylation activate the pheromone response pathway reporter *FUS1*. First, partial loss-of-function mutants that compromise early (*pgi1*, *pmi40*, *psa1*, and *dpm1*) or late (*och1*, *alg1*, *mnn10*, and *spt14*) steps in protein glycosylation stimulate expression of *FUS1* in *ste4* mutants. We suggest that mutations in other genes required for protein glycosylation, such as *SEC53* and genes required for O-linked glycosylation, would also stimulate *FUS1* transcription, although these possibilities have not been tested. Second, supplementation with mannose can suppress both the slow-growth and *FUS1* transcription phenotypes of *pgi1-101* and *pmi40-101* mutants, presumably by providing an alternate route to synthesis of mannose-6-P. Third, multicopy plasmid suppressors of the growth defects of *dpm1-101* mutants (*PSA1* and *RER2*) also suppressed *FUS1* expression. We have isolated multicopy suppressors of the growth defect of *pmi40-101* cells, and these also significantly reduce *FUS1* expression (P. J. Cullen and G. F. Sprague, unpublished results). Fourth, tunicamycin, which prevents N-linked glycosylation, stimulates *FUS1* transcription. It has been shown that defects in protein glycosylation also induce the unfolded protein response (UPR; for review see Sidrauski *et al.* 1998), which results in an accumulation of endoplasmic reticulum-specific chaperones. Although we favor the hypothesis that protein glycosylation defects lead to Sho1 activation, it is conceivable that the UPR influences *FUS1* transcription in the protein glycosylation mutants.

The results of Lee and Elion (1999) also support a function for *STE* pathway components in protein glycosylation mutants. Our results are largely consistent with theirs but differ in three notable points. First, we have shown that defects in any part of the protein glycosylation pathway (not simply *OCH1*) lead to activation of the pheromone response pathway (see Figure 1). Second, we have shown that Sho1, an osmosensor in the HOG pathway, is an essential component of this Ste-dependent signaling cascade (Table 6). Third, we have shown that pathway activation is not constitutive, but rather dependent upon the osmotic environment of the cell, an observation that is consistent with the requirement for the Sho1 osmosensor in this pathway (Table 3 and see below).

Components from the HOG, pheromone response, and invasive growth pathways are required for signaling in protein glycosylation mutants: Activation of *FUS1* transcription in protein glycosylation mutants requires proteins known to operate in one or more signal transduction pathways: the pheromone response pathway, the HOG pathway, and the invasive growth pathway. On the basis of the known relationships of these proteins, we suggest the following amalgamated pathway: Sho1-Ste20/Ste50-Ste11-Ste7-Kss1-Ste12 (see Figure 5). This genetic pathway most closely resembles the invasive growth pathway, especially since Sho1 has been implicated in filamentous growth (O'Rourke and Herskowitz 1998). For example, KSS1 is known to be exclusively required for the invasive pathway (Cook *et al.* 1997; Madhani *et al.* 1997), and we have shown that in protein glycosylation mutants activation of *FUS1* transcription is also mediated by *KSS1* and not *FUS3*, consistent with observations by Lee and Elion (1999). However, the two pathways differ in several important respects. First, Hsl7 and Tec1, which are involved in the invasive growth pathway (Fujita *et al.* 1999 and Madhani *et al.* 1997, respectively), have no discernible effect on *FUS1* transcription in protein glycosylation mutants. Second, activation of the invasive growth response has not been observed to activate *FUS1* transcription (Madhani *et al.* 1997; our unpublished observations). Third, the glycosylation mutants do not exhibit physiological changes associated with activation of the invasive growth pathway, namely elongated cell morphology and unipolar budding pattern. Likewise, although the pathway we have deduced includes proteins that function in HOG and pheromone response, not all proteins from these pathways are required for the transcription effect we observe. Specifically, Ste4, Ste5, and Fus3 from the pheromone pathway and Pbs2 from the HOG pathway are not required.

The requirement of Sho1 for *FUS1* transcription in protein glycosylation mutants is notable for several reasons. First, a link from Sho1 through Ste7, Kss1, and Ste12 to *FUS1* transcription has not been observed previously in cells containing wild-type pathways, although cross talk from the HOG pathway to *FUS1* transcription can occur in *pbs2* and *hog1* mutants (O'Rourke and Herskowitz 1998). Second, the requirement for Sho1, a predicted glycosylated membrane protein, provides a possible mechanism by which defects in protein glycosylation activate *FUS1* transcription. Defects in glycosylation lead to changes in the osmotic sensitivity of the cell, thereby activating Sho1. Whether *FUS1* (and other pheromone responsive genes) is an intended target when Sho1 is activated in this way or whether its activation represents loss of pathway specificity is not known. Third, the requirement for *SHO1* provides an explanation as to why Ste11 is in both the pheromone and HOG pathways: to simultaneously activate both pathways under special conditions (see below).

Osmosensitivity in protein glycosylation mutants causes Sho1-dependent activation of *FUS1*: Defects in protein glycosylation components result in an osmosensitive cell (*e.g.*, Stateva *et al.* 1991). We have been able to partially rescue the slow-growth phenotype of protein glycosylation mutants (including *pmi40-101* and *och1*Δ) by the addition of salt (NaCl or KCl) to the medium. Addition of salt caused a concomitant reduction in *FUS1-lacZ* activity of more than fivefold (not shown). In addition, growth of mutants in YPD caused a more severe growth defect in protein glycosylation mutants, which correlates with an increase in expression of *FUS1-lacZ*. Thus, it appears that *FUS1* expression responds to the general osmotic environment in these mutants. These observations may also rationalize why loss of a target of the protein glycosylation pathway, *GAS1*, causes activation of *FUS1*, since *gas1* mutants are known to have severe cell wall and osmolarity defects (Popolo *et al.* 1997). Together, these results lead us to hypothesize that Sho1 is activated in response to the osmotic stress these mutants suffer.

Defects in protein glycosylation lead to Sho1-dependent activation of the pheromone-responsive *FUS1* gene, whereas other treatments that activate Sho1—addition of osmolyte to the medium or nutrient limitation—do not have this effect. We do not know the reason for this difference, but two possibilities merit consideration. One possibility is that the glycoslyation defect in some way causes a loss of pathway specificity and in this sense the effect on *FUS1* transcription is unintended. For example, mutation of Hog1 allows cross talk between the HOG and pheromone pathways (O'Rourke and Herskowitz 1998). Perhaps Hog1 is inactivated in the protein glycosylation mutants. We suggest, however, that the effect is intended. Among the genes whose transcription is activated in response to pheromone are *FKS2* (a component of β-1,3-glucan synthase; Mazur *et al.* 1995) and *CHS1* (a chitin synthase; Appeltauer and Achstetter 1989; Diaz *et al.* 1992; Philips and Herskowitz 1997; Cappellaro *et al.* 1998), which function in cell wall biosynthesis. Increased expression of these genes in protein glycosylation mutants would provide one means for the cell to compensate for the cell wall defect. Indeed, increased *FKS2* expression has been observed in *och1* mutants (Lee and Elion 1999). Ste12 may be targeted to promoters of cell wall genes in part by heterodimerization with Mcm1, a DNA-binding protein whose targets include cell wall genes and whose phosphorylation state is sensitive to osmolarity (Kuo *et al.* 1997). Thus, the Sho1-Ste12 link provides a way to tie changes in cell wall structure/function detected by Sho1 to expression of genes needed to strengthen or remodel the cell wall. Furthermore, it seems likely that this link is important physiologically in wild-type cells given the synthetic interactions we observe between Sho1-Ste12 pathway mutations and protein glycosylation mutations. Thus, the Sho1-Ste12 pathway may be a signaling path-

way that communicates changes in protein glycosylation and thereby adjusts accordingly the transcription of cell wall integrity components.

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