The Signaling Mucins Msb2 and Hkr1 Differentially Regulate the Filamentation Mitogen-activated Protein Kinase Pathway and Contribute to a Multimodal Response

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A central question in the area of signal transduction is why pathways utilize common components. In the budding yeast *Saccharomyces cerevisiae*, the HOG and filamentous growth (FG) MAPK pathways require overlapping components but are thought to be induced by different stimuli and specify distinct outputs. To better understand the regulation of the FG pathway, we examined FG in one of yeast's native environments, the grape-producing plant *Vitis vinifera*. In this setting, different aspects of FG were induced in a temporal manner coupled to the nutrient cycle, which uncovered a multimodal feature of FG pathway signaling. FG pathway activity was modulated by the HOG pathway, which led to the finding that the signaling mucins Msb2p and Hkr1p, which operate at the head of the HOG pathway, differentially regulate the FG pathway. The two mucins exhibited different expression and secretion patterns, and their overproduction induced nonoverlapping sets of target genes. Moreover, Msb2p had a function in cell polarization through the adaptor protein Sho1p that Hkr1p did not. Differential MAPK activation by signaling mucins brings to light a new point of discrimination between MAPK pathways.

INTRODUCTION

Signal transduction pathways regulate the cellular response to diverse stimuli. Signaling pathways typically function in highly connected networks where multiple inputs become integrated into a global response (Bhattacharyya *et al.*, 2006). For example, mitogen-activated protein kinase (MAPK) pathways utilize overlapping or shared components to coordinate different aspects of cellular behaviors (Bardwell, 2006). The overlap between pathways can be extensive, and it remains unclear how a particular signal transmitted through an interconnected network elicits a specific response. Given that inappropriate cross talk between MAPK pathways is an underlying cause of cancer and other diseases (Santen *et al.*, 2002), understanding how signaling pathways precisely coordinate cellular behaviors is an important question.

In the budding yeast *Saccharomyces cerevisiae*, MAPK pathways regulate the response to a variety of stimuli (Errede *et al.*, 1995). In response to nutrient limitation, cells undergo filamentous growth (FG; pseudohyphal/invasive growth;

Gimeno *et al.*, 1992; Liu *et al.*, 1993; Roberts and Fink, 1994; Cullen and Sprague, 2000), a cellular differentiation characteristic of many fungal species including pathogens (Lo *et al.*, 1997; Whiteway and Bachewich, 2007). FG is regulated by a typical MAPK pathway (Roberts and Fink, 1994; Borneman *et al.*, 2007). At the head of the FG pathway, the signaling mucin Msb2p and adaptor protein Sho1p (O'Rourke and Herskowitz, 1998; Cullen *et al.*, 2004) connect to the polarity establishment Rho (Ras homology) GTPase Cdc42p (Peter *et al.*, 1996; Leberer *et al.*, 1997), a global regulator of cell polarity and signaling (Johnson, 1999). In its activated (GTP-bound) state, Cdc42p associates with the p21-activated kinase (PAK) Ste20p (Peter *et al.*, 1996; Leberer *et al.*, 1997), which results in the activation of a typical MAPK cascade composed of the Ste11p, Ste7p, and Kss1p protein kinases in a phosphorelay circuit (Madhani *et al.*, 1997). Phosphorylation of the transcription factors Ste12p and Tec1p by Kss1p results in the induced expression of FG pathway target genes (Madhani and Fink, 1997; Madhani *et al.*, 1999). Although it is not entirely clear how nutritional information connects to FG pathway signaling, activation of the FG pathway requires processing and release of the extracellular inhibitory domain of Msb2p by the aspartyl protease Yps1p, which occurs preferentially under nutrient-limiting conditions (Vadaie *et al.*, 2008).

Most of the proteins that function in the FG pathway are required for the activation of other MAPK pathways in the same cell (see Figure 1; Schwartz and Madhani, 2004; Qi and Elion, 2005; Murphy and Blenis, 2006). Msb2p, Sho1p, Cdc42p, Ste20p, Ste11p (O'Rourke and Herskowitz, 1998; O'Rourke *et al.*, 2002; Tatebayashi *et al.*, 2006) and the Ste11p-adaptor protein Ste50p (Posas *et al.*, 1998; Jansen *et al.*, 2001; Truckses *et al.*, 2006) function in the FG pathway and the high osmolarity glycerol response (HOG) pathway (Raitt *et*

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Abbreviations used: FG, filamentous growth; FRE, filamentation response element; GEF, guanine nucleotide exchange factor; GFP, YFP, and CFP, green, yellow, and cyan fluorescent protein, respectively; HA, hemagglutinin; HOG, high osmolarity glycerol response; MAPK, mitogen-activated protein kinase; ORF, open reading frame; PAK, p21 activated kinase; PM, plasma membrane; SC, synthetic complete.

al., 2000; Reiser *et al.*, 2000; Tatebayashi *et al.*, 2007), which controls the response to osmotic stress (Hohmann, 2002; Chen and Thorner, 2007). The Ste11p branch of the HOG pathway converges with the Sln1p branch at the level of the MAPKK Pbs2p (Maeda *et al.*, 1994). A second mucin, Hkr1p, functions redundantly with Msb2p in the HOG pathway (see Figure 1; Tatebayashi *et al.*, 2007). Whether Hkr1p also functions in the FG pathway is not known. Cdc42p, Ste20p, Ste50p, Ste11p, and Ste7p also function in the pheromone

response pathway, which controls mating of complementary haploid cells that sense and respond to secreted peptide pheromones (Bardwell, 2004; Schwartz and Madhani, 2004; Dohlman and Slessareva, 2006). The three MAPK pathways are activated by different stimuli and induce different target genes and morphological responses (Roberts *et al.*, 2000; McClean *et al.*, 2007). Specificity between the FG and HOG pathways is regulated at least in part at the level of Pbs2p, which binds to Sho1p and Ste11p, presumably to recruit

Table 1. Yeast strains

Strain	Genotype	Source
PC538 ^a	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52</i>	Cullen <i>et al.</i> (2004)
PC986 ^b	<i>MATa ura3-52 leu2 his3 trp1</i>	Roberts <i>et al.</i> (2000)
BY4741 ^c	<i>his3Δ0 leu2Δ0 ura3Δ0 met15Δ0</i>	Research Genetics, Huntsville, AL
PC560	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 snf1::URA3</i>	Cullen and Sprague (2000)
PC544	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bni1::URA3</i>	Cullen and Sprague (2002)
PC563	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud8::URA3</i>	Cullen and Sprague (2002)
PC999	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA</i>	Cullen <i>et al.</i> (2004)
PC586	<i>MATα ura3-52 leu2</i>	Cullen <i>et al.</i> (2004)
PC1531	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sho1::HYG</i>	Cullen <i>et al.</i> (2004)
PC948	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 msb2::KanMX6</i>	Cullen <i>et al.</i> (2004)
PC1702	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 SHO1-HA::KanMX6</i>	Vadaie <i>et al.</i> (2008)
PC622	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1::KanMX6</i>	Cullen <i>et al.</i> (2004)
PC1083	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-MSB2-HA::KanMX6</i>	Cullen <i>et al.</i> (2004)
PC2559	<i>PIL1-mCherry::HisMX</i>	Walther <i>et al.</i> 2006
PC1516	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2^{Δ100-818}</i>	Vadaie <i>et al.</i> (2008)
PC2043	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 FLO11-HA</i>	Unpublished data ^d
PC313	<i>MATa ura3-53</i>	Cullen and Sprague (2000)
PC318	<i>MATa ura3-53 petite</i>	This study
PC549	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::URA3</i>	This study
PC562	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ras2::URA3</i>	This study
PC539	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::URA3</i>	This study
PC1029	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KanMX6</i>	This study
PC2053	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pbs2::KanMX6</i>	This study
PC1519	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pgu1::KanMX6</i>	This study
PC2687	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ste12::URA3</i>	This study
PC2689	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA ras2::URA3</i>	This study
PC2063	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 HOG1-GFP::KanMX6</i>	This study
PC1502	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-PBS2::KanMX6</i>	This study
PC1523	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ssk1::NAT</i>	This study
PC2061	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ssk1::NAT ste11::URA3</i>	This study
PC2625	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 hkr1::HYG</i>	This study
PC2662	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 hkr1::HYG sho1::URA3</i>	This study
PC2664	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 hkr1::HYG msb2::URA3</i>	This study
PC2668	<i>MATα ura3-52 leu2 hkr1::HYG</i>	This study
PC2740	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 HKR1-HA</i>	This study
PC2746	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-HKR1</i>	This study
PC1748	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 SHO1-GFP::KanMX6</i>	This study
PC2666	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 hkr1::HYG MSB2-HA</i>	This study
PC1983	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ABP140-YFP::HYG SHO1-YFP::KanMX6</i>	This study
PC1866	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ABP140-YFP::HYG</i>	This study
PC1983	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ABP140-CFP::HYG SHO1-YFP::KanMX6</i>	This study
PC624	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1 ste12::URA3</i>	This study
PC2850	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 hkr1::HYG sho1::URA3 msb2::NAT</i>	This study
PC670	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-SHO1 pbs2::URA3</i>	This study
PC3059	<i>MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 GAL-PBS2::KanMX6 hog1::NAT</i>	This study
PC2519	<i>MATa ste4 FUS1-lacZ::NAT FUS1-HIS3 ura3-52</i>	This study
PC2110	<i>MATa ste4 FUS1-lacZ::NAT FUS1-HIS3 ura3-52 pbs2::KanMX6</i>	This study
PC2522	<i>MATa ste4 FUS1-lacZ::NAT FUS1-HIS3 ura3-52 sho1::KanMX6</i>	This study
PC2184	<i>MATα leu2::ura3 ste12::URA3</i>	This study
PC2545	<i>MATa ste4 FUS1-lacZ::NAT FUS1-HIS3 ura3-52 ste12::KanMX6</i>	This study

^a All strains are Σ 1278b background unless otherwise indicated.

^b S288c background ordered deletion collection control strain.

^c Deletion strains from the *MATa* ordered deletion collection in the S288c background were also used in this study.

^d Karunanithi, S., Vadaie, N., Chavel, C., Dionne, H. M., Joshi, J., Birkaya, B., Grell, L., and Cullen, P. J.

these proteins to the HOG pathway (Maeda *et al.*, 1995; Zarrinpar *et al.*, 2004).

To better understand the FG pathway, we examined FG in settings that mimic the native environments of this organism. Budding yeast exists in a variety of natural settings, including as a pathogen in immunocompromised patients (McCusker *et al.*, 1994), but is commonly found in the wild associated with the grape-producing plant *Vitis vinifera*. Extensive cultivation over thousands of years has reinforced the relationship between the two organisms (Cavalieri *et al.*, 2003). We therefore developed a “grape assay” that among other things uncovered a multimodal FG response mediated by the FG pathway. Communication between the HOG and FG pathways led to modulation of the FG response, and we specifically show that the signaling mucins Msb2p and Hkr1p exert different effects on FG pathway activity. Our findings therefore bring to light a new point of discrimination between MAPK pathways by members of the signaling mucin family of proteins. More generally, our findings suggest a possible rationale for why MAPK pathways share components, to coordinate a cellular response through mutually exclusive activation between pathways.

MATERIALS AND METHODS

Strains, Plasmids, and Microbiological Techniques

Yeast strains are listed in Table 1 and plasmids in Supplemental Table S1. Yeast and bacterial strains were manipulated by standard methods (Sambrook *et al.*, 1989; Rose *et al.*, 1990). All experiments were carried out at 30°C unless otherwise indicated. β -Galactosidase assays were performed as described (Cullen *et al.*, 2000) and represent at least two independent trials. The single cell invasive growth assay (Cullen and Sprague, 2000) and the plate-washing assay (Roberts and Fink, 1994) were performed to evaluate FG. The pGAL-inducible plasmid containing the *PBS2^{DD}* allele was generously provided by H. Saito (University of Tokyo, Tokyo, Japan) (Wurgler-Murphy *et al.*, 1997). Plasmids containing FG pathway targets fused to *lacZ* and plasmid V84 were provided by C. Boone (University of Toronto, Toronto, Canada) (Roberts *et al.*, 2000). Reporters included *lacZ* fusions to a filamentation response element (FRE; Madhani *et al.*, 1997), *MSB2*, a transcriptional target that encodes an upstream regulator of the pathway (Cullen *et al.*, 2004), *KSS1*, which encodes the MAPK (Figure 1; Madhani and Fink, 1997); *PGU1*, which encodes a secreted enzyme that hydrolyzes polygalacturonic acid, present in plant cell walls (Madhani *et al.*, 1999), and *YLR042c*, which encodes a protein of unknown function (Roberts *et al.*, 2000). Plasmid pIL30-URA3 containing

FgTy-*lacZ* was provided by B. Errede (University of North Carolina, Chapel Hill, NC) (Laloux *et al.*, 1994), and pFRE-*lacZ* was provided by H. Madhani (University of California, San Francisco, San Francisco, CA) (Madhani *et al.*, 1997). Overexpression constructs were obtained from an ordered collection obtained from Open Biosystems (Huntsville, AL; Gelperin *et al.*, 2005). Gene disruptions and *GAL1* promoter fusions were made by PCR-based methods (Baudin *et al.*, 1993; Longtine *et al.*, 1998), including the use of antibiotic resistant markers (Goldstein and McCusker, 1999) and epitope fusions (Schneider *et al.*, 1995). Integrations were confirmed by PCR analysis and phenotype. Internal epitopes were at 298 residues for the Hkr1p protein, and 500 residues for the Msb2p protein. The *FUS1-lacZ* reporter (typically a mating pathway reporter) was used to evaluate the activity of the FG pathway for some experiments, which in Σ 1278b strains lacking an intact mating pathway exhibits Msb2p-, Sho1p-, and Ste12-dependence (Cullen *et al.*, 2004). *FUS1-HIS3* expression was used to confirm *FUS1-lacZ* reporter data and was measured by spotting equal amounts of cells onto synthetic medium lacking histidine and containing 4-amino-1,2,4-triazole. Budding pattern was based on established methodology (Chant and Pringle, 1995) and was confirmed for some experiments by visual inspection of connected cells. Endopolygalacturonase activity of strains was assessed using the method adapted from Gaijovs *et al.* (1994), except that equal concentrations of cells were spotted onto synthetic complete (SC) media containing 6.7 g/l of yeast nitrogen base without amino acid, 2% glucose, 1% polygalacturonic acid (Fluka, Ronkonkoma, NY), and 50 mM potassium phosphate buffer (pH 8.0). Plates were incubated for 2 d at 30°C. After incubation plates were flooded with 1% ruthenium red (Sigma, St. Louis, MO) overnight to ensure uniform staining. Plates were washed with a gentle stream of distilled water and photographed. The *RCK2* gene was identified in a genetic screen using an inducible plasmid library (AYES library; Ramer *et al.*, 1992). Plasmids were transformed into a wild-type strain (PC538), and >10,000 colonies were examined by replica plating from S-GAL-URA medium to S-GAL-URA-HIS medium to identify those that failed to express the *FUS1-HIS3* reporter and failed to grow. A single plasmid-dependent isolate containing *RCK2* (PC1460) was identified, which was confirmed by retesting. Plasmid mutagenesis of pSHO1-GFP was performed as described (Vadaie *et al.*, 2008).

Assays Involving *V. vinifera*

For most experiments, $\sim 1 \times 10^5$ yeast cells were applied to 0.5-cm lateral sections of *V. vinifera* berries macerated with a wooden applicator. Yeast cells applied to sectioned grapes grew robustly on macerated sections more so than unperturbed sections. Inoculated tissues were incubated at 30°C. Pilot experiments using different inoculation strategies and sources of *V. vinifera* gave similar outcomes. To confirm that yeast cells in *V. vinifera* arose from input cells, strains contained plasmids carrying genes that encoded fluorescently tagged proteins (pSHO1-GFP URA3 in pRS316; Sikorski and Hieter, 1989) or pEB0876 pho4NLS-GFP in pRS316 (Strawn *et al.*, 2004). An osmometer (5100B Vapor Pressure Osmometer, Wescor, Logan, UT) was used to determine the osmolality of *V. vinifera* extracts, and Glucostix were used to estimate glucose concentrations.

For growth assays, yeast strains were inoculated onto halved and skinned *V. vinifera* tissues in sterile tubes containing 0.5 ml of water. Cultures were incubated for 24 h at 30°C, the mixture was vortexed briefly, and serial dilutions were plated onto synthetic media. Cell adherence was determined similarly except that nonadherent cells were removed by washing extracts. To examine the colonization of *V. vinifera*, inoculated extracts were examined at 96 h by embedding in 2% agarose, based on established procedures (Ruzin, 1999). Tissues embedded in agarose were cut into sections and incubated in 5% paraformaldehyde for 16 h. Paraformaldehyde-treated tissues were embedded in tissue-sectioning holders using Optimal Cutting Temperature (OCT) Compound (Tissue-Tek, Product No. 4583, Sakura, Torrance, CA) at -20°C. Approximately 15–20- μ m lateral tissue sections were taken using a cryostat (Riechert-Jung, Walldorf, Germany; Cryocut 1800). Tissue sections were immediately placed onto ice-cold microscope slides and observed by microscopy. The FG pathway was not required for growth under laboratory conditions, in filtered *V. vinifera* extracts (not shown), or in high-glucose medium (Supplemental Table S2), which indicates that the defect does not arise from the high glucose levels found in this environment.

DNA Microarray Analysis

Wild-type (PC538), *GAL-MSB2* (PC1083), and *GAL-HKR1* (PC2746) strains were induced in YEP-GAL medium for 6 h. At the 6-h time point, cells were examined by microscopy for the characteristic filamentation response. RNA was prepared by hot acid phenol and passage over an RNeasy column (Qiagen, Chatsworth, CA). DNA microarray analysis was performed as described (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997). Microarray construction, target labeling, and hybridization protocols were as described (Fazzio *et al.*, 2001). Sample comparisons were independently replicated at least three times, each of which was derived from a separate induction. Fluoro-reverse experiments were used to identify sequence-specific dye biases. Arrays were scanned using a GenePix 4000 scanner (Axon Instruments, Burlingame, CA). Image analysis was performed using GenePix Pro3.0. Array features (i.e., spots) having low signal intensities or signals compromised by artifacts were

Figure 1. The FG and HOG MAP kinase pathways. The two pathways are shown with factors that function in both pathways in black, pathway-specific factors for the FG pathway in green, and HOG pathway, red. The Sln1p branch of the HOG pathway is also shown. The Ste7p and Ste12p proteins are shown in black because they are required for activation of the FG and pheromone response pathways.

Figure 2. The colonization of grapes by *S. cerevisiae*. (A) Wild-type (PC538), *ste12Δ* (PC539), and *pbs2Δ* (PC2053) strains were grown to saturation in synthetic medium, and equal concentrations of cells were applied to sectioned grape discs and incubated for 72 h at 30°C. The experiment was performed more than three times, and a representative image is shown. Bar, 1 cm. (B) Cells on a sectioned grape disk after a 24-h incubation visualized by microscopy at 20×. Bar, 200 μm. (C) Wild-type (PC538, panels 1 and 2) and *ste12Δ* cells (PC539, panel 3) were extracted from grapes and examined by microscopy at 100×. Representative cells are shown. Bar, 5 μm. The arrow refers to grape tissue attached to a yeast cell. (D) Yeast cells enmeshed in grape tissue. Sectioned grape discs were incubated with wild-type (PC538) or *flo11Δ* (PC1029) cells for 16 h. Samples were briefly vortexed to remove nonadherent cells and examined by microscopy at 100× in a focal series through the plane of the Z-axis. Bar, 20 μm. Top panels, a merged DIC rhodamine image. The grape tissue appears red because of an autofluorescent signal. Bottom panels, the position of yeast cells determined by visual inspection of serial sections through the plane of the Z-axis. Supplemental Movies 1 and 2 show the complete Z-stack. Quantitation of the adherence data are presented in Table 2. (E) Yeast colonization of grape tissue. 3D rendering of serial sections through the plane of the Z-axis of a merged dark field and rhodamine image at 20× magnification. Yeast cells appear light, and a subset of the grape tissues red due to an autofluorescent signal. Individual channel images are shown in Supplemental Figure S1, D–H. Bar, 200 μm.

removed from further analysis. Background subtracted Cy5/Cy3 ratios were $\log_2$ transformed and a loess normalization strategy ($f = 0.67$) was applied for each array using S-Plus (MathSoft, Cambridge, MA). Each feature where the $|\log_2(\text{ratio})| \geq 0.8$, the corresponding gene was considered differentially expressed.

Genomic Screening Approach

The *MATa* haploid deletion collection containing ~4800 haploid strains (Gaeffer *et al.*, 2002) was transferred to YEPD medium and YEPD medium containing 1.4 M glucose, 1 M sorbitol, or 1 M KCl. Plates were incubated for 4 d at 30°C. Deletion strains were manipulated using a 96-fixed pin pinning tool (V&P Scientific, San Diego, CA; VP 408) and a plate replication tool (V&P Scientific, VP 381) in Omnitrays (VWR International, Bridgeport, NJ.). For growth-curve analysis, strains were diluted in 96-well format in 250 μl water and pinned to YEPD, YEPD + 1.4 M glucose, and YEPD + 0.9 M NaCl liquid media. Growth curves were performed at 0, 2, 4, 6, 8, 24, 48, and 72 h using a Spectra MR spectrophotometer (Dynex, Richfield, MN). Microscopic examination of glucose-sensitive mutants showed a range of phenotypes in high-glucose medium including cell lysis, hyperpolarized growth, or no phenotype indicative of a contribution from multiple pathways in the response (data not shown). Process/function and GO annotations were made using *Saccharomyces* Genome Database (Cherry *et al.*, 1998; Hong *et al.*, 2008).

Microscopy

Differential interference contrast (DIC) and fluorescence microscopy using rhodamine, FITC, and yellow and cyan fluorescent protein (YFP and CFP, respectively) filter sets were performed using an Axioplan 2 fluorescent microscope (Zeiss, Thornwood, NY) with a Plan-Apochromat 100×/1.4 (oil) objective (NA 0.17). Digital images were obtained with the Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition and analysis and for rendering 3D Z-stack images. Images were further analyzed in Adobe Photoshop (San Jose, CA), where adjustments of brightness and contrast were made.

Protein Analysis

Immunoblots were performed as described (Cullen *et al.*, 2004). Proteins were separated by SDS-PAGE on 10% precast gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (protran BA85, VWR International, Bridgeport, NJ). Membranes were incubated in blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20) for 1 h at 25°C. ECL Plus immunoblotting reagents were used to detect secondary antibodies (Amersham Biosciences, Piscataway NJ). Nitrocellulose membranes were incubated for 18 h at 4°C in blocking buffer containing a primary rabbit polyclonal IgG antibody against dually phosphorylated p38 (9211S, Cell Signaling Technology, Beverly, MA) or a mouse monoclonal IgG antibody against green fluorescent protein (GFP; Roche Diagnostics, Mannheim, Germany) to detect Hog1p-GFP. Phosphotyrosine assays were performed based on the following protocol (Reiser *et al.*, 2000). A wild-type strain

Table 2. Colonization, adherence, and FG of yeast strains in *V. vinifera*

Strain	Relevant genotype	Colonization ^a	Adherence ^b	FG (%) ^c
PC538	Wild type	1×10^6	1	50
PC539	<i>ste12Δ</i>	3.2×10^5	0.45 ± 0.1	1
PC562	<i>ras2Δ</i>	3.5×10^5	0.6 ± 0.1	5
PC1029	<i>flo11Δ</i>	3.8×10^5	0.35 ± 0.1	50
PC2053	<i>pbs2Δ</i>	1.1×10^1	ND	ND

ND, not determined.

^a Colonization was determined by serial dilutions of overnight cultures containing inoculated *V. vinifera* tissues. The number of cells was extrapolated from the number of colonies that grew on plates after incubation at 30°C for 24 h.

^b Adherence of the indicated strains was determined by visual examination of cells inoculated overnight in liquid cultures containing *V. vinifera* tissue. The relative proportion of associated cells was compared to wild-type cells, which was arbitrarily assigned the value of 1.

^c FG was assessed by microscopic examination of yeast cells removed from inoculated grapes after 48 h. More than 300 cells were examined for each strain. The percentage indicates the number of cells observed in the filamentous form compared to the total number of cells.

(PC538) and isogenic strain containing *HOG1-GFP* (PC2063) were grown to midlog phase in YEPD media at 30°C. Cells were harvested by centrifugation and resuspended in YEPD, YEPD + 1.4 M glucose, YEPD + 1 M sorbitol, or *V. vinifera* extracts at 30°C for 30 min. Cells were harvested and resuspended in 8 M urea, 5% SDS, and 1% β -mercaptoethanol, 0.1 mM sodium metavanadate (Sigma), and Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics). After the addition of glass beads, cells were disrupted by vortexing, and protein extracts were separated by SDS-PAGE analysis. Protein alignments were made using ClustalW (European Bioinformatics Institute, Cambridge, United Kingdom), and protein domain determinations were made using the Prosite database (ExPASy; Swiss Institute of Bioinformatics).

RESULTS

An Assay to Examine FG in Grapes

We determined if yeast undergoes FG in grapes and whether the response resembles that observed under standard laboratory conditions. Inoculation of sectioned grape discs with yeast cells resulted in growth (Figure 2A, wild type). Full colonization required the FG pathway (*ste12Δ*,

Figure 2A). Microscopic examination showed a colonization defect in FG pathway mutants (Figure 2B) that was confirmed by quantitation of cell numbers in grapes (Table 2). Yeast cells underwent FG in grapes (Figure 2C, panel 1; Table 2). Filamentous cells were observed ~48 h after inoculation, presumably when nutrients had become limiting, which resulted in a mixed population of yeast-form and filamentous-form cells (Figure 2C, panels 1 and 2; and see Supplemental Figure S1C). The FG pathway was required for the cellular differentiation to the filamentous morphology in grapes (Figure 2C, panel 3; Table 2).

Yeast cells adhered to grape tissue (Figure 2C, panel 2, arrow) and became enmeshed at high levels (Figure 2D, Supplemental Movie 1). Adherence was dependent on the FG pathway (Table 2) and cell-surface flocculin Flo11p (*flo11Δ*, Figure 2D; Supplemental Movie 2; Table 2), which is a target of the pathway that controls cell-cell and cell-substrate adherence (Guo *et al.*, 2000; Lambrechts *et al.*, 1996;

Figure 3. The FG pathway induces a multimodal response. (A) Wild-type (PC538), *ste12Δ* (PC539), and *flo11Δ* (PC1029) cells were incubated in different concentrations of glucose (GLU; 25%, 2%, and 0.2%), or 2% galactose (GAL) for 8 h and examined by microscopy at 100×. Stationary, SCD medium, 24 h. More than 10,000 cells were examined for each condition over three separate trials, and a representative image is shown. Bar, 25 μ m. (B) Wild-type (PC538, top colony), *pgu1Δ* (PC1519, bottom left colony), and *ste12Δ* (PC539, bottom right colony) cells were spotted onto synthetic medium containing polygalacturonic acid supplemented with 2, 8, 16, or 20% glucose (GLU) and assayed for pectinase activity at 24 h. Far left, cells were removed from the wild-type spot on the 2% GLU plate to show that Pgu1p-secreting cells are in the yeast form. Bar, 5 μ m. (C) The plate-washing assay. Strains were spotted onto YEPD plates supplemented with 2, 8, 16, or 20% glucose (GLU) and incubated for 72 h at 30°C. The plates were photographed (left panel), washed in a stream of water, and photographed again (right panels). The full time series is presented in Supplemental Figure S2. Wild-type (PC538) and *ras2Δ* (PC562), top line; *ste12Δ* (PC539) and *flo11Δ* (PC1029), line 2; *snf1Δ* (PC560) and *pgu1Δ* (PC1519), line 3; and *bni1Δ* (PC544) and *bud8Δ* (PC563), bottom line. (D) Transcriptional reporters of the FG pathway were examined in a wild-type strain (PC586) or a *ste12* mutant (PC2184) incubated in synthetic medium supplemented with 0.2, 2, or 20% glucose or 2% galactose (G) for 4 h. β -Galactosidase assays were performed in duplicate, and the average of two experiments is shown. Error bars, SD between trials. Values are presented in Miller units (U).

Lo and Dranginis, 1996, 1998; Reynolds and Fink, 2001). Unexpectedly, adherence and enmeshment were mediated predominately by yeast-form cells (Figure 2D). As a result, efficient colonization of specific tissues such as veins, tissues, and cavities by yeast-form cells was observed at early time points after inoculation (16 h), as measured by 3D rendering of inoculated grape discs (Figure 2E). Therefore, yeast undergoes a filamentation response in grapes that involves multiple steps in a temporal sequence coordinated by the FG pathway.

The FG Pathway Induces a Multimodal Response

We reasoned that different aspects of FG might be induced at different nutrient levels. To test this possibility, outputs of the FG pathway were examined at different glucose concentrations, given that glucose depletion is a potent inducer of FG (Cullen and Sprague, 2000). Outputs of the FG pathway include Flo11p-dependent adhesion (Rupp *et al.*, 1999), cell elongation (Kron *et al.*, 1994; Madhani *et al.*, 1999), unipolar budding (Gimeno *et al.*, 1992; Cullen and Sprague, 2002), agar invasion (Roberts and Fink, 1994), and secretion of the plant cell wall degrading pectinase Pgu1p (Madhani *et al.*, 1999). Flo11p- and Ste12p-dependent cell-cell adherence was observed between yeast-form cells at high concentrations of glucose (Figure 3A, 20% GLU) and was more apparent at lower glucose concentrations (Figure 3A, 2%, 0.2% GLU). Stationary phase cultures did not exhibit detectable cell-cell adherence (Figure 3A, Stationary). Pectinase secretion was similarly observed from yeast-form cells (Figure 3B). Pectinase secretion was stimulated at lower glucose concentrations (Figure 3B). Agar invasion followed a similar trend (Figure 3C). The appearance of filamentous cells, which exhibit unipolar budding and elongated cell morphologies, was observed at much lower concentrations of glucose (Figure 3A, 0.2% GLU) consistent with previous observations (Cullen and Sprague, 2000).

The above results indicate that the FG pathway induces different aspects of FG at different glucose concentrations. Consistent with this possibility, transcriptional (*lacZ*) reporters of the FG pathway were expressed over a range of glucose concentrations (Figure 3D). Conditions optimal for FG (YEP-GAL medium) caused a modest increase (1.5–3-fold depending on the reporter) in FG pathway activity (Figure 3D; G, green bars), which corresponded to enhanced cell elongation (Figure 3A, GAL) and elevated processing of Msb2p by its cognate protease (Vadaie *et al.*, 2008). Therefore, the FG pathway induces different aspects of FG in a sequence coupled to the nutrient-growth cycle. The response involves a linear increase in some outputs (adherence and pectinase) and stepwise induction of others (cell elongation and unipolar budding). This complex modality is consistent with the observed colonization of grapes by yeast cells.

The HOG Pathway Contributes to the Modality of FG Pathway Signaling

We undertook a genomics approach to identify genes required for growth in high-glucose environments similar to those encountered in grapes (Conde *et al.*, 2006). Approximately 90 mutants were identified that were required for growth in YEPD + 25% glucose that were ranked by strength-of-phenotype using growth-curve analysis (Figure 4A; Supplemental Table S2). Among the mutants that showed the strongest defect were those lacking an intact HOG pathway (*pbs2Δ* and *hog1Δ*; Figure 4A; Supplemental Figure S3A). The *gpd1Δ* mutant was also identified (Figure 4A), which is a HOG pathway target required for glycerol production (Albertyn *et al.*, 1994). Genes that function in a

Figure 4. The HOG pathway modulates the activity of the FG pathway. (A) Graph of the growth of mutants defective for growth in YEPD + 25% glucose at the 48-h time point. HOG pathway mutants *hog1Δ*, *pbs2Δ*, and *gpd1Δ* are highlighted in red and labeled. X-axis, mutants; Y-axis, OD (absorbance at 600 nm). The OD of wild-type cells is marked by the dotted line. The complete dataset is presented in Supplemental Table S2. (B) The *pbs2Δ* mutant exhibits glucose- and osmotic stress-dependent cell polarization. Wild-type (PC538) and *pbs2Δ* (PC2053) cells were grown in YEPD or YEPGAL media supplemented with 0.5 M glucose (GLU) or 0.5 M KCl. (C) Overexpression of *PBS2* (*GAL-PBS2*; PC1502) inhibits agar invasion (left) and FG, as assessed by the single-cell invasive growth assay (right). Bar, 5 μm. (D) *KSS1-lacZ* expression in strains overexpressing HOG pathway components. β-Galactosidase assays were performed as described in Figure 3D.

nutritional capacity were also identified (Supplemental Table S2). A role for the HOG pathway in this context is not surprising given that high concentrations of dissolved sugars would be expected to create an osmotic imbalance. We

confirmed that the HOG pathway is activated at high concentrations of glucose based on the phosphorylation (Supplemental Figure S3B) and nuclear localization (Supplemental Figure S3C) of the MAPK for the HOG pathway Hog1p (Maeda *et al.*, 1995; Ferrigno *et al.*, 1998). The HOG pathway was required for growth on grapes (*pbs2Δ*, Figure 2, A and B, Table 2) and was activated to ~80% its maximal level within minutes of exposure to this environment (Supplemental Figure S3D). High glucose represents a commonly encountered osmotic stress that connects the HOG pathway to the nutrient cycle of this organism.

The HOG pathway might modulate FG pathway activity in high glucose, given that the two pathways require overlapping components (Figure 1) and that high concentrations of glucose activate the HOG pathway (Supplemental Figure S3) and inhibit FG (Figure 3). This possibility fits with the general idea that the HOG and FG pathways function in mutually exclusive activation states (Davenport *et al.*, 1999; O'Rourke *et al.*, 2002; Westfall and Thorner, 2006), although the relationship between the pathways has not been thoroughly examined. Deletion of the *PBS2* gene caused hyperinvasive growth (Supplemental Figure S4) and hyperpolarized growth that was stimulated by increasing concentrations of glucose (Figure 4B, YEPD + 0.5M GLU). The *pbs2Δ* mutant showed elevated pectinase activity (Supplemental Figure S5A) and elevated expression of the FG pathway reporter *FRE-lacZ* (Supplemental Figure S7B). Likewise, overexpression of *PBS2* inhibited invasive growth, cell polarization, pectinase levels, and *FRE* reporter expression (Figure 4C and Supplemental Figure S5A). Osmotic stresses KCl and sorbitol similarly inhibited FG (Supplemental Figure S4). Inhibition of FG by glucose or osmotic stress was more severe than seen in FG pathway mutants (Supple-

mental Figure S4, cf. *ste12Δ* mutant YEPD 10 d with wild-type cells YEPD + 1 M KCl 10 d), indicating that other mechanisms may contribute to the inhibition of FG that extend beyond the FG pathway. Progressive repression of FG by glucose in a concentration-dependent manner is indicative of the gradually sloping activation of the HOG pathway by increasing osmolyte (Hersen *et al.*, 2008; Supplemental Figure S7A).

Inhibition of the FG pathway might occur at the level of the Pbs2p-Sho1p and Pbs2p-Ste11p interactions (Maeda *et al.*, 1995; Zarrinpar *et al.*, 2004). Bypass of the inhibitory effect of *GAL-PBS2* (Supplemental Figure S5, B and C) and osmotic stress (Supplemental Figure S6) was observed using hyperactive alleles *MSB2*^{Δ100–818} (Vadaie *et al.*, 2008), *SHO1*^{P120L} (Vadaie *et al.*, 2008), and *STE11-4* (Stevenson *et al.*, 1992). Because the alleles do not affect protein levels or presumably interfere with the Pbs2p interaction (Stevenson *et al.*, 1992; Vadaie *et al.*, 2008), the bypass is not likely mediated by altered association of the proteins with Pbs2p. The inhibitory effect of *GAL-PBS2* or an activated allele *PBS2*^{DD} (Wurgler-Murphy *et al.*, 1997) required the MAPK Hog1p (Supplemental Figure S7, C and D, respectively), further suggesting that the HOG pathway exerts its effect through downstream MAPK signaling (i.e., targets). Overexpression of many HOG pathway components dampened FG pathway activity, including Ypd1p, Ssk1p, Ssk2p, Ssk22p, Pbs2p, Hog1p, and the transcription factor Hot1p (Figure 4D). We also identified the calmodulin-like protein kinase Rck2p, which is a target of Hog1p (Bilsland-Marchesan *et al.*, 2000; Teige *et al.*, 2001) in a genetic screen for genes that dampen the FG pathway when overexpressed (Figure 4D). Taken together, our results are consistent with an inhibitory role for the

Figure 6. Expression profiling in cells overexpressing *HKR1* or *MSB2*. (A) Genes significantly induced in cells overexpressing *HKR1*. (B) Genes significantly induced in cells overexpressing *MSB2*. (A and B) Heat maps generated from DNA microarray data

HOG pathway on the FG pathway that is potentially mediated at multiple levels.

Msb2p and Hkr1p Differentially Activate the FG Pathway

The signaling mucin Hkr1p functions redundantly with Msb2p in the HOG pathway (Tatebayashi *et al.*, 2007), but its role in the FG pathway has not been examined. To determine whether Hkr1p functions in the FG pathway, the *HKR1* gene was disrupted in a wild-type strain of the $\Sigma 1278b$ background and assessed for FG and MAPK activity. The *hkr1* Δ mutant did not show a defect in agar invasion (Figure 5A), whereas the *msb2* Δ mutant was defective for invasive growth (Figure 5A; Cullen *et al.*, 2004). Deletion of *HKR1* in an *msb2* Δ mutant restored agar invasion (Figure 5A), which indicates that Hkr1p negatively regulates the FG pathway in this context. Deletion of *HKR1* in the *sho1* Δ mutant failed to restore agar invasion (Figure 5A), indicating that the inhibitory effect of Hkr1p is mediated through Sho1p. The expression of FG pathway reporters corroborated the invasive growth phenotypes (Figure 5B). Overexpression of *HKR1* did not induce agar invasion (Figure 5C), unlike overexpression of *MSB2*, which caused hyperinvasive growth (Figure 5D; Cullen *et al.*, 2004). In fact, overexpression of *HKR1* dampened MAPK activity in cells carrying activated alleles of *MSB2* and *SHO1* (Figure 5D). Together, these results demonstrate that Hkr1p is not a component of the FG pathway and has an inhibitory effect on the pathway in certain genetic contexts.

Expression profiling was used to confirm the genetic data. Overexpression of *MSB2* and *HKR1* induced nonoverlapping target genes by DNA microarray analysis (Figure 6, Supplemental Table S3). Specifically, overexpression of *HKR1* induced the expression of ~ 35 genes, 14 of which are targets of the HOG pathway (Figure 6A, HOG; O'Rourke and Herskowitz, 2004; Posas *et al.*, 2000). Overexpression of *HKR1* did not induce FG pathway targets. Overexpression of *MSB2* induced the expression of ~ 30 genes that include FG pathway targets (Figure 6B, FG; Madhani *et al.*, 1999; Roberts *et al.*, 2000) and genes involved in nutritional scavenging (Figure 6B, S) but not HOG pathway targets. Msb2p-dependent induction of FG pathway targets might be underrepresented because under this condition (YEP-GAL) FG targets are induced in wild-type cells.

Msb2p and Hkr1p Exhibit Distinct Expression and Secretion Patterns

Msb2p and Hkr1p are members of the signaling mucin family of proteins, cell-surface glycoproteins that regulate Ras- and Rho-dependent pathways (Carraway *et al.*, 2003; Singh and Hollingsworth, 2006). Msb2p and Hkr1p represent the only members of that family in *S. cerevisiae* (Laura Grell and P. J. Cullen, unpublished observations). Like other signaling mucins, Msb2p and Hkr1p are single-pass transmembrane proteins that contain tandem S/T/P-rich repeats in their extracellular domains (Figure 7A). Despite the similarity in overall topology, the proteins exhibit little se-

show differential expression of genes induced in response to overexpression of *HKR1* (GAL-*HKR1*) or overexpression of *MSB2* (GAL-*MSB2*). Red, induced genes; green, repressed genes; yellow, no change; gray, no signal. Expression data were collected from three separate experiments derived from independent inductions. The values for each experiment are shown. FG, FG pathway target; HOG, HOG pathway target; S, nutritional scavenging. The complete dataset is presented in Supplemental Table S3.

Figure 7. Expression and secretion analysis of the Hkr1p and Msb2p proteins. (A) The *MSB2* and *HKR1* ORFs and upstream regulatory sequences. Scale bar, 100-amino acid residues (aa). The Ste12p and Tec1p binding sites in the *MSB2* promoter are labeled. The asterisk refers to the point mutation in the Ste12p-binding site (A→G –479 from transcriptional start site). Regions of sequence homology (47% identity by ClustalW analysis) are shown in yellow, which overlaps the cleavage domain of Msb2p (CD) and may demark the potential CD for Hkr1p (CD?). Regions of the proteins that show <20% homology are shown in green for Msb2p and red for Hkr1p. The mucin homology domain (MHD), transmembrane domain (TM), and signal peptide (SP) domains are also labeled. The S/T-rich regions are shown in purple. LZ, leucine zipper. (B) Wild-type (PC2519), *sho1Δ* (PC2522), and *ste12Δ* (PC2545) strains containing the *MSB2-lacZ*, *MSB2^{AG}-lacZ*, and *HKR1-lacZ* fusions were grown to saturation in SD-URA medium. Cells were collected by centrifugation, washed twice, and resuspended in SD-URA, S-GAL-URA, or SD-URA medium supplemented with 1 M KCl. Cells were grown for 5 h, except for the S-GAL-URA culture which was grown for 16 h to induce maximal FG pathway activation. β -Galactosidase assays were performed as described in Figure 3D. (C) Colony immunoblot of Hkr1p-HA (PC2740), Msb2p-HA (PC999), and Sho1p-HA (PC1702) strains. Cells were patched onto YEPD medium overlaid with nitrocellulose filters and incubated for 48 h at 30°C. The membrane was photographed (top panel) and washed in a stream of water to remove cells. Membranes were probed with anti-HA antibodies (bottom panel). (D) The S/P ratios of the Msb2p-HA, Hkr1p-HA, and Sho1p-HA proteins was determined by standard immunoblot and colony immunoblot analysis derived from cells grown in YEPD, YEP-GAL, or YEPD + 1M sorbitol (1M Sorb) medium for 24 h. Ratios were determined by taking into account S and P volumes.

sequence similarity in their open reading frames (ORFs) or upstream regulatory domains (Figure 7A).

To further compare the two mucins, the expression patterns of the *MSB2* and *HKR1* genes were examined under different conditions. The *HKR1* gene is expressed at low levels (Kasahara *et al.*, 1994), and an *HKR1-lacZ* fusion was expressed at lower levels than an *MSB2-lacZ* fusion (Figure 7B). *HKR1-lacZ* expression was modestly induced by osmotic stress (Figure 7B), whereas *MSB2-lacZ* expression was somewhat reduced (Figure 7B). *MSB2-lacZ* expression was induced under nutrient-limiting conditions that promote FG (Figure 7B). The *MSB2* promoter contains upstream regulatory elements for the transcription factors Tec1p and Ste12p (Figure 7A), and its expression is induced by an autophed-back loop (Cullen *et al.*, 2004). Starvation-dependent induction of *MSB2-lacZ* was dependent on Sho1p and Ste12p (Figure 7B) and was not observed using a construct that contains a point mutation in the Ste12p-consensus binding site (*MSB2^{AG}-lacZ*; Figure 7, A and B). The upstream regulatory sequence of the *HKR1* promoter does not contain FG pathway regulatory elements (Figure 7A), and the *HKR1-lacZ* reporter did not show Sho1p or Ste12p-dependent induction (Figure 7B) or induction by nutrient limitation (Figure 7B). Published expression profiling data closely agree with these results (O'Rourke and Herskowitz, 2004).

Msb2p is processed as part of its activation mechanism, and the glycosylated extracellular domain of the protein is

shed from cells (Vadaie *et al.*, 2008). Colony blot analysis showed that the extracellular domain of an epitope-tagged Hkr1p-hemagglutinin (HA) fusion was also shed (Figure 7C). Processing of Msb2p correlates with its activity and is elevated under glucose-limiting conditions (Figure 7D; Vadaie *et al.*, 2008). Shedding of Hkr1p-HA was not induced by nutrient limitation (Figure 7D). Osmotic shock did not cause a boost in secretion of either mucin (Figure 7D); however, osmotic shock may induce the formation of cell-surface vesicles that trap shed proteins (see below), which complicates the interpretation of this result. The expression and secretion profiles of the two mucins are consistent with the idea that they primarily function in different pathways.

Msb2p Induces Sho1p-Dependent Polarization, Whereas Hkr1p Does Not

Msb2p and Hkr1p function through the adaptor Sho1p (Tatebayashi *et al.*, 2007; Vadaie *et al.*, 2008) and may exert different effects on the Sho1p protein. To test this possibility, we first examined the properties of the Sho1p protein with respect to FG and HOG pathway activation. Sho1p contains four membrane-spanning domains and a cytoplasmic SH3 domain. Biochemical analysis showed that the protein migrates as a dimer (Supplemental Figure S8) as previously reported (Hao *et al.*, 2007) and mutational analysis identified a residue (S149F) adjacent to the tetraspan domain that was

Figure 8. Msb2p and Hkr1p differentially influence cell polarization through Sho1p. (A) Localization of Sho1p-GFP in synthetic medium supplemented with glucose (SCD) or 2% galactose (SC-Gal). (B) Localization of Sho1p-GFP and Bud8p-GFP as indicated in SD medium with or without 2 M sorbitol (2M Sorb) in the indicated

required for the Sho1p-Sho1p interaction (Supplemental Figure S8). The Sho1p-Sho1p interaction was not affected by nutrient limitation, osmotic stress, or the activation state of the protein (Supplemental Figure S8), which indicates that the oligomerization of Sho1p may not underlie its specification between pathways.

The localization of Sho1p was examined in response to nutrient limitation and osmotic stress. In nutrient-rich conditions, Sho1p-GFP was localized preferentially to buds as previously reported (Figure 8A; Raitt *et al.*, 2000; Reiser *et al.*, 2000). Under nutrient-limiting conditions, Sho1p-GFP localization to highly polarized sites (Figure 8A) was dependent on the SH3 domain of the protein (Supplemental Figure S10). In response to osmotic shock, Sho1p-GFP localized to punctate sites (Figure 8B) that did not require the SH3 domain or *Pbs2p* (Figure 8B). The punctate pattern is indicative of global invaginations in the plasma membrane (PM; Slaninova *et al.*, 2000), and *Sln1p*, *Msb2p*, and *Hkr1p* have been reported to localize to similar sites in response to osmotic shock (Reiser *et al.*, 2003; Tatebayashi *et al.*, 2007). Indeed, other cell-surface proteins like *Bud8p*-GFP showed a similar punctate pattern in response to osmotic stress (Figure 8B). Structural deconvolution followed by 3D rendering confirmed that Sho1p-GFP was localized to cell-surface sites that were stable with respect to each other and the cell periphery (Supplemental Figure S9; Supplemental Movies 3 and 4). The punctate sites did not overlap with eisosomes, static sites for endocytosis of receptor complexes (Walther *et al.*, 2006), as visualized by colocalization with an eisosome-specific marker (Figure 8C).

The localization pattern of Sho1p is consistent with the fact that during FG cells undergo polarized growth, whereas in response to osmotic stress cells do not polarize, in part through actin cytoskeleton disassembly (Chowdhury *et al.*, 1992; Lillie and Brown, 1994; Yuzyuk *et al.*, 2002; Yuzyuk and Amberg, 2003). The localization of Sho1p to punctate sites was independent of the depolarization of the actin cytoskeleton (Figure 8D). The localization of Sho1p to punctate sites and concomitant depolarization of the actin cytoskeleton may contribute to the lack of Sho1p- and MAPK-dependent polarization during osmotic stress.

Msb2p interacts with the polarity establishment GTPase *Cdc42p* (Cullen *et al.*, 2004). In cells overexpressing *MSB2*, cells were hyperpolarized (Figure 8E). This phenotype was partly independent of the MAPK pathway but required Sho1p (Figure 8E). Overexpression of *Hkr1p* inhibited the hyperpolarized growth of cells containing *GAL-MSB2* (Figure 8E), whereas deletion of *HKR1* had the opposite effect (Figure 8E). The two mucins also induced different localization patterns of Sho1p-GFP, likely due to their differential effects on cell polarization (Supplemental Figure S10). The inhibitory effect of *Hkr1p* on cell polarization may or may

genetic contexts; *ΔSH3*, Sho1p-*ΔSH3*-GFP. (C) Sho1p-GFP does not colocalize with the eisosome-specific marker *Pil1p*-RFP. (D) *Abp140p*-YFP (PC1866) and Sho1p-*ΔSH3*-GFP localization in latrunculin A (Lat A)- and sorbitol-treated cells. Cells containing *Abp140p*-YFP localizes to actin cables (Yang and Pon, 2002). DMSO was used in the control (Ctl) localization. Sho1p-*ΔSH3*-GFP was used in this experiment because of its even distribution at the cell surface. Cells were pretreated with Lat A for 30 min. Where indicated, cells were treated with 2 M sorbitol (Sorb) for 5 min. (E) Hyperpolarized growth was measured in the indicated strains. Cells were considered hyperpolarized if the long axis was >1.5-fold longer than the short axis. More than 200 cells grown in SC-GAL medium were measured, and the average of two independent experiments is shown. Error bars, SD between experiments.

not be direct, as activation of the HOG pathway by other means exerts a similar effect (Supplemental Figure S10). Therefore, Msb2p can be functionally distinguished from Hkr1p by its ability to induce cell polarization through Sho1p.

DISCUSSION

In this study we explored MAPK regulation from a unique perspective, by examining the foraging behavior of *S. cerevisiae* in one of its native environments, grapes produced by the plant *V. vinifera*. This approach yielded a wealth of information, including new insights into the different roles that signaling mucins play in discrimination between MAPK pathways and a multimodal response induced by the FG pathway. Our results validate the approach of exploring MAPK-dependent responses in “natural” settings to bring to light new features of MAPK regulation.

Different Signaling Mucins Function Preferentially in Different MAPK Pathways

We have identified a potentially new point of discrimination between the FG and HOG pathways at the level of the signaling mucins Msb2p and Hkr1p. This discovery represents an important contribution, because Hkr1p is the first HOG pathway component to be identified at the pathway's head that does not also function in the FG pathway (among the Msb2p, Sho1p, Cdc42p, Ste20p, Ste50p, and Ste11p proteins; Figure 1). A straightforward possibility supported by this study is that Msb2p functions primarily in the FG pathway, whereas Hkr1p functions mainly in the HOG pathway. Msb2p appears to function as a dedicated component of the FG pathway, based in its ability to induce cell polarization, its expression and secretion profiles, and its processing, which occurs under nutrient-limiting conditions and is required for activation of the FG pathway (Vadaie *et al.*, 2008). Msb2p might functionally substitute for Hkr1p in the HOG pathway (Tatebayashi *et al.*, 2007) in a manner that is analogous to the MAPKs Fus3p and Kss1p, which function primarily in the mating and FG pathways, respectively, but functionally substitute for each other if one MAPK is absent (Madhani *et al.*, 1997).

Likewise, Hkr1p appears to function as a HOG pathway-specific factor. Hkr1p is not required for FG pathway signaling and when overexpressed does not induce FG pathway targets. Expression of the *HKR1* gene is not induced by nutrient limitation and is not under the control of the FG pathway. In some settings, Hkr1p inhibits the FG pathway at the level of Sho1p. Taken together, our results indicate that the two mucins function preferentially in different MAPK pathways. This conclusion may slightly oversimplify the biological roles of the two mucins, as their overexpression also induces target genes (such as nonoverlapping genes involved in cell wall biosynthesis, mitochondrial function, and sporulation) that have not been established as targets of either MAPK pathway.

It is not yet clear how Msb2p and Hkr1p mediate their different outputs. An attractive possibility is they recruit different proteins to the cell surface through their cytoplasmic signaling domains, which bear little similarity to each other. The cytoplasmic tail of Msb2p associates directly with Cdc42p and contributes to cell polarization. Hkr1p contains a distinctly different cytoplasmic domain that includes a leucine zipper motif. Both proteins interact with Sho1p, and the conditional inhibition of Sho1p by Hkr1p in cells lacking Msb2p might be explained if the mucins compete for a binding site in the Sho1p protein. Discrimination between

different pathway outputs may extend to other members of the signaling mucin family. The mammalian mucin MUC4 imparts specificity in MAPK pathway outputs through differential polarization of the ErbB2 receptor (Ramsauer *et al.*, 2006).

Multimodality of the Filamentation MAPK Pathway

We have found that the FG pathway induces different aspects of FG at different nutrient levels. This conclusion is supported by the fact that different aspects of FG are genetically separable (Cullen and Sprague, 2002) and resolves an established paradox with respect to nutrition and FG pathway signaling. FG is potentially triggered by glucose depletion (Cullen and Sprague, 2000; Kuchin *et al.*, 2003), yet the pathway is active in nutrient-rich environments (Cullen *et al.*, 2004; Madhani *et al.*, 1999), in protein glycosylation mutants (Cullen *et al.*, 2000), in mutants that exhibit cross talk (O'Rourke and Herskowitz, 1998), and during mat-form growth (Reynolds and Fink, 2001). The fact that the FG pathway does not exist in ON and OFF states but is active throughout the nutrient-growth cycle unifies these apparently paradoxical observations.

Other MAPK pathways, like the pheromone response pathway, similarly exhibit multimodality (Hao *et al.*, 2008; Moore, 1983). A unique aspect of multimodality in FG pathway regulation is by its inhibition mediated by the HOG pathway, which shares components with the FG pathway but functions in a mutually exclusive manner. HOG pathway-dependent inhibition of the FG pathway accounts for one aspect of pathway modality at high concentrations of glucose (>20–2%). Regulatory feed ins at other glucose concentrations may occur through nutrient-regulatory pathways such as the RAS pathway (Mosch *et al.*, 1996) and the ATP/AMP kinase Snf1p (Hedbacker and Carlson, 2008). In a broad sense, our findings might provide an explanation for why the FG and HOG MAPK pathways have maintained the sharing of common components over evolutionary time, to create mutually exclusive activation states that function in graded opposition as part of a coordinated behavior. Multimodality resulting from exclusive inhibition may extend to other MAPK pathways that share components.

Yeast Foraging and Fungal Pathogenesis

Fungal pathogens remain a significant threat to many aspects of human health (Sanchez and Larsen, 2007), in part because their behaviors in complex environments like metazoan tissues remains largely unclear (Chandra *et al.*, 2005; Lo *et al.*, 1997). By examining yeast foraging in a complex natural setting, we show that adherence and penetration functions occur before dimorphism. Some pathogenic fungi like *Cryptococcus neoformans* exhibit invasive properties and virulence in the yeast form (Lin and Heitman, 2006), which underscores the potency of these poorly understood processes in pathogenic efficacy. Pathogens moreover induce target genes in a spatiotemporal hierarchy by the induction of early and late genes (Schlumberger and Hardt, 2006). Likewise, budding yeast induces distinct facets of FG in a spatiotemporal hierarchy coupled to the nutrient-growth cycle. Future studies of yeast behaviors in natural settings will build on the overall understanding of fungal foraging that might aid in our appreciation of fungal pathogenesis.

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