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Mid2 Is a Putative Sensor for Cell Integrity Signaling in *Saccharomyces cerevisiae*

MATHUMATHI RAJAVEL,[1] BEVIN PHILIP,[1] BENJAMIN M. BUEHRER,[2] BEVERLY ERREDE,[2] AND DAVID E. LEVIN[1]*

Department of Biochemistry, School of Public Health, The Johns Hopkins University, Baltimore, Maryland 21205,[1] and Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599[2]

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Hcs77 is a putative cell surface sensor for cell integrity signaling in *Saccharomyces cerevisiae*. Its loss of function results in cell lysis during growth at elevated temperatures (e.g., 39°C) and impaired signaling to the Mpk1 mitogen-activated protein kinase in response to mild heat shock. We isolated the *MID2* gene as a dosage suppressor of the cell lysis defect of an *hcs77* null mutant. *MID2* encodes a putative membrane protein whose function is required for survival of pheromone treatment. Mid2 possesses properties similar to those of Hcs77, including a single transmembrane domain and a long region that is rich in seryl and threonyl residues. We demonstrate that Mid2 is required for cell integrity signaling in response to pheromone. Additionally, we show that Mid2 and Hcs77 serve a redundant but essential function as cell surface sensors for cell integrity signaling during vegetative growth. Both proteins are uniformly distributed through the plasma membrane and are highly O-mannosylated on their extracellular domains. Finally, we identified a yeast homolog of *MID2*, designated *MTL1*, which provides a partially redundant function with *MID2* for cell integrity signaling during vegetative growth at elevated temperature but not for survival of pheromone treatment. We conclude that Hcs77 is dedicated to signaling cell wall stress during vegetative growth and that Mid2 participates in this signaling, but its primary role is in signaling wall stress during pheromone-induced morphogenesis.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (3, 20). The cell must remodel this rigid structure during vegetative growth and during pheromone-induced morphogenesis. Wall remodeling is monitored and regulated by the cell integrity signaling pathway controlled by the Rho1 GTPase. Two essential functions have been identified for Rho1. First, it serves as an integral regulatory subunit of the 1,3-β-glucan synthase (GS) complex that stimulates GS activity in a GTP-dependent manner (7, 32). A pair of closely related genes, *FKS1* and *FKS2*, encode alternative catalytic subunits of the GS complex (6, 13, 28, 33) that are the presumed targets of Rho1 activity.

A second essential function of Rho1 is to bind and activate protein kinase C (19, 29), which is encoded by *PKC1* (26, 39). Loss of *PKC1* function, or of any of the components of the mitogen-activated protein (MAP) kinase cascade under its control (25), results in a cell lysis defect that is attributable to a deficiency in cell wall construction (23, 24, 31). The MAP kinase cascade is a linear pathway comprised of a MEK kinase (*BCK1* [4, 22]), a pair of redundant MEKs (*MKK1/2* [14]), and a MAP kinase (*MPK1* [21]). One of the consequences of signaling through the MAP kinase cascade is transcriptional activation of *FKS2* (41).

Cell integrity signaling is induced in response to several environmental stimuli. First, signaling is activated persistently in response to growth at elevated temperatures (e.g., 37 to 39°C [18]), consistent with the finding that null mutants in many of the pathway components display cell lysis defects only when cultivated at high temperatures. Second, hypo-osmotic shock induces a rapid but transient activation of signaling (5, 18). Finally, treatment with mating pheromone stimulates signaling at a time that is coincident with the onset of morphogenesis (1, 8). Indeed, mutants defective in cell integrity signaling undergo cell lysis during pheromone-induced morphogenesis (8).

The mechanism by which information regarding the state of the cell wall is transmitted to the intracellular signaling apparatus remains an open question. Recently, a gene encoding a putative cell surface sensor for the activation of cell integrity signaling was described and variously designated *HCS77* (9), *WSC1* (38), and *SLG1* (16). Loss of function of *HCS77* results in a cell lysis defect that is less severe than that observed for other components of the cell integrity signaling pathway (9, 16, 38), suggesting that its function may be partially redundant. Indeed, several *HCS77* homologs (*WSC2, -3*, and *-4*, exist in budding yeast. *WSC2* and *WSC3* have been reported to contribute to cell integrity signaling to a lesser degree than *HCS77* (38). In our hands, mutants in these genes in combination with *hcs77* have only a modest effect on the severity of the cell lysis defect. Moreover, residual signaling to the Mpk1 MAP kinase is evident even in an *hcs77 wsc2 wsc3* triple mutant (38), indicating that these signaling components serve a partially redundant function with another unidentified sensor. Here we present evidence that the Mid2 protein is a cell surface sensor for cell integrity signaling, which together with Hcs77 provides an essential function during vegetative growth. Additionally, we demonstrate that Mid2 is required for activation of this signaling pathway in response to pheromone-induced morphogenesis.

MATERIALS AND METHODS

Strains, growth conditions, and transformations. The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cultures were grown in YEPD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose). Synthetic minimal (SD) medium (34) supplemented with the appropriate nutrients was used to select for plasmid maintenance and gene replacement. Yeast transformations were carried out by

* Corresponding author. Mailing address: Department of Biochemistry, The Johns Hopkins University, School of Public Health, 615 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-9825. Fax: (410) 955-2926. E-mail: levin@welchlink.welch.jhu.edu.

TABLE 1. *S. cerevisiae* strains used

Strain	Relevant genotype	Reference or source
1783	*MAT***a**[a]	I. Herskowitz
1784	*MAT*α[a]	I. Herskowitz
1788	*MAT***a**/*MAT*α[a]	I. Herskowitz
DL1235	*MAT*α *rho1-5*[b] (YOC755)	19
DL1985	*MAT***a***hcs77*Δ::*LEU2*[a]	This study
DL1987	*MAT***a**/*MAT*α *hcs77*Δ::*LEU2*[a]	This study
DL2040	*MAT***a** *hcs77*Δ::*LEU2 SUP1*[a]	This study
DL2230	*MAT***a**/*MAT*α *hcs77*Δ::*LEU2 wsc2*Δ::*LEU2*[a]	This study
DL2256	*MAT***a**/*MAT*α *hcs77*Δ::*LEU2*/*HCS77*[a]	This study
DL2277	*MAT***a** *mid2*Δ::*URA3*[a]	This study
DL2282	*MAT***a** *mid2*Δ::*URA3 hcs77*Δ::*LEU2*[a]	This study
DL2337	*MAT***a**/*MAT*α *mid2*Δ::*URA3*/*MID2*[a]	This study
DL2355	*MAT***a** *mtl1*Δ::*HIS4*[a]	This study
DL2357	*MAT***a** *mid2*Δ::*URA3 mtl1*Δ::*HIS4*[a]	This study
DL2394	*MAT***a**/*MAT*α *mid2*Δ::*URA3*[a]	This study
DL2395	*MAT***a**/*MAT*α *mtl1*Δ::*HIS4*[a]	This study
DL2396	*MAT***a**/*MAT*α *mid2*Δ::*URA3 mtl1*Δ::*HIS4*[a]	This study
DL2393	*MAT***a** *cdc28-13*[c] (D13au)	1
DL2435	*MAT***a** *cdc28-13 mid2*Δ::*URA3*[c]	This study

[a] EG123 background: *leu2-3,112 trp1-1 ura3-52 his4 can1*.
[b] YPH500 background: *ura3-52 lys2-801 ade2-101 trp-*Δ*63 his3-*Δ*200 leu2-*Δ*1*.
[c] D13au background: *ade1 his2 leu2-3,112 trp1-1a ura3*Δ*NS*.

the lithium acetate method (15). *Escherichia coli* DH5α was used for the propagation of all plasmids. *E. coli* cells were cultured in Luria broth medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl) and transformed by standard methods (27).

Isolation of *MID2* and *MTL1*. *S. cerevisiae* DL1987 (*hcs77*Δ) was transformed with a plasmid library of genomic yeast DNA (from strain DL2040) in centromeric vector pRS316 (35), constructed as described previously (22). Transformants were grown at room temperature and replicated onto YEPD at 38°C for 2 days. Plasmids were rescued from colonies arising at the nonpermissive temperature. Clone pRM5.2 contained an 8.4-kb insert that included three complete open reading frames (ORFs). In this clone, the *MID2* gene was flanked by YLR331C and *RPS31*. A *Bgl*II-*Bam*HI subclone that possesses *MID2* and YLR331C was suppression competent. A 3′ truncation of *MID2* (by *Sna*BI) eliminated the suppression activity of this clone.

The *MTL1* gene was amplified by PCR from wild-type (1783) genomic DNA with 1.0 kb of 5′ flanking sequence and 290 bp of 3′ flanking sequence and then cloned into the 2μm-based plasmid pRS424 (35). The DNA sequence of the complete insert was determined. DNA sequence analysis was performed by the JHU Biosynthesis and Sequencing Facility, using oligonucleotides synthesized by the Biochemistry Core Facility. PCRs for the purposes of cloning were carried out with *Pfu* polymerase (Stratagene); *Taq* polymerase (Perkin-Elmer) was used for analytical purposes (see below). The sequence of *MTL1* from strain 1783 contained 84 additional nucleotides compared with the database sequence from S288C. These residues result in an in-frame insertion of 28 amino acid residues (nearly all seryl) at position 265, which is within the Ser/Thr-rich region of this protein (see Fig. 5).

Genomic deletions of *MID2* and *MTL1*. For construction of the *mid2*Δ allele, plasmid pRS315[*mid2*Δ::*URA3*] (provided by H. Iida) was digested with *Xho*I and *Ssp*I, and the resultant 1.6-kb fragment was purified and used to transform yeast strains DL2256 and DL2393. Transformants of DL2256 were sporulated, and tetrad analysis was carried out on YEPD with or without 10% sorbitol. Deletion of *MID2* was confirmed by PCR in both strains.

To delete the genomic copy of *MTL1*, a 900-bp fragment that is immediately 5′ to the *MTL1* coding sequence, amplified by PCR from genomic 1783 DNA, was first inserted into pBluescript[*HIS4*] (gift of Susan Michaelis). Next, a 610-bp fragment that includes 320 bp of coding sequence and 290 bp immediately 3′ to the *MTL1* coding sequence was amplified and inserted into the resultant plasmid on the other end of *HIS4* in the same orientation as the 5′ sequence. This construct, designated p*mtl1*Δ::*HIS4*, was digested with *Bss*HII to liberate a 6.4-kb fragment, which was used to transform yeast strain DL2337. Deletion of *MTL1* was confirmed by PCR.

Pheromone-induced killing. To test for sensitivity to killing by α-factor, *MAT***a** strains were grown in synthetic minimal medium containing limiting calcium (100 μM $CaCl_2$) (12) for 24 h, subcultured in the same medium, and grown to an A_{600} of 0.5 to 1.0 (5×10^6 to 1×10^7 cells/ml). Cells were then treated with α-factor (8 μg/ml; Sigma), and viability was measured over time by plating onto YEPD.

Construction of *MID2* and *HCS77* fusions to green fluorescent protein (GFP) and the hemagglutinin (HA) epitope. Mid2 was tagged at its C terminus with a UV-optimized variant of GFP, GFPuv. In the first of two steps, the GFPuv coding sequence (711 bp, including the stop codon) was amplified by PCR from pGFPuv (Clontech), with three additional guanosyl residues at the 5′ end. This fragment was blunt-end inserted into the *Sma*I site of pRS315 (35) so as to regenerate a single *Sma*I site immediately 5′ of the GFPuv sequence. Next, a 1.9-kb fragment including *MID2* with 760 bp of 5′ noncoding sequence (amplified from pRM5.2) but without its stop codon was blunt-end inserted into the *Sma*I site of pRS315[GFPuv]. This construct (p*MID2*-GFP) expressed GFPuv fused to the C terminus of Mid2 with an additional glycyl residue at the fusion point.

Mid2 and Hcs77 were tagged at their C termini with the 3xHA (three-repeat HA) epitope (40). In the first of two steps, the 3xHA sequence from *PKC1-HA* (39) was amplified together with 250 bp of 3′ noncoding sequence from the *PKC1* transcriptional terminator and inserted into the *Sma*I site of YEp352 (10) so as to regenerate a single *Sma*I site immediately 5′ to the epitope sequence. Next, *MID2* (same fragment as above) and *HCS77* sequences (including 600 bp 5′ to the translational start site) were amplified through the final coding base (excluding the stop codon), and blunt-end inserted into the *Sma*I site of YEp352[3xHA]. The resultant clones fused the 3xHA epitope, in frame with an additional glycine residue, to the C termini of Mid2 and Hcs77.

Construction of N-glycosylation site mutants. Hcs77-HA N-glycosylation mutants N4Q and N65Q were constructed by site-directed mutagenesis using the PCR overlap extension method (11). Pairs of complementary mutagenic primers (AAATAATGAGACCGCAAAAAACAAGTCTGC and its reverse complement for the N4Q mutation; CTTTGCCCTTTATCAACATTCAGAATGTTA and its reverse complement for the N65Q mutation) were used in separate PCRs with primers either 5′ or 3′ to the coding sequence to generate overlapping 5′ and 3′ regions of *HCS77*-HA, using YEp352[HCS77-HA] as the template. The products of those reactions were combined in a third reaction to amplify the full-length mutated alleles, which were then digested with *Sac*I and *Xba*I and inserted into the cognate sites of YEp352. Construction of the double mutant was done by generating the N65Q mutation in the N4Q mutant.

Fluorescence microscopy of Mid2-GFP. Haploid strain DL2277 expressing Mid2-GFP from pRS315 was grown in SD medium for 24 h, subcultured in YEPD to an A_{600} of 0.5 to 1.0, and washed three times with phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2OPO_4 [pH 7.4]). The cells were resuspended in Vectashield mounting medium (Vector Laboratories, Inc.) with Hoechst 33342 (10 μg/ml). A Zeiss fluorescence microscope fitted with a fluorescein isothiocyanate filter was used to visualize cells.

Activation of Mpk1-HA by temperature shift and pheromone treatment. Strains expressing Mpk1-HA were tested for thermal activation (18) or pheromone-induced activation (1) of this protein kinase by immunoprecipitation of the fusion protein from cell extracts with monoclonal antibody 12CA5 (BabCo and Boehringer Mannheim), followed by immunocomplex protein kinase assays. Strains were tested for thermal activation of Mpk1-HA as described previously (18). Strains tested for pheromone-induced activation were grown to a density of ca. 10^7 cells/ml at room temperature in SD medium. Cells were harvested and resuspended in prewarmed (to 37°C) YEPD containing 100 mM sorbitol. The cultures were incubated at this temperature for 1.5 to 2 h until the cells, which bear a temperature-sensitive *cdc28* allele, accumulated at the G_1 block as large unbudded cells. At this time ($t = 0$), samples of each culture were removed for preparation of protein extracts (50 ml), mating pheromone was added to the remaining culture (50 nM α-factor), and the cultures were incubated at 37°C. Samples (50 ml) were removed at the indicated times following pheromone addition for preparation of extracts. Extracts were prepared and protein concentrations were determined as described previously (1). Immunocomplex protein kinase assays were performed as described elsewhere (18).

α-Mannosidase treatment. Mid2-HA and Hcs77-HA were immunoprecipitated from cell extracts as described previously (18) except that 100 μg of protein was used and the immunoprecipitation buffer contained 1% Triton X-100 instead of Nonidet P-40. Immunoprecipitates were washed twice with the mannosidase assay buffer (100 mM sodium acetate [pH 5.0], 2 mM $ZnCl_2$) and resuspended in 15 μl of buffer. α-Mannosidase (5 μl of 0.1 U/μl) was added to the immunocomplexes and incubated for 3 h at 37°C. Controls were mock treated with assay buffer only under the same conditions. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% polyacrylamide gel followed by immunoblotting as described previously (18). Protein concentrations of cell extracts that contained detergent were determined by using the bicinchoninic acid protein assay reagent (Pierce).

Fractionation of Hcs77-HA and Mid2-HA. Transformants of yeast strain 1788 expressing either Mid2-HA or Hcs77-HA from YEp352 were grown in YEPD to an A_{600} of 1 to 1.2. Cells were harvested from 400 ml, washed once with 10% sorbitol, and resuspended in 6 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 50 mM KF) with protease inhibitors (20 μg of leupeptin, 20 μg of benzamidine, 10 μg of pepstatin, and 40 μg of aprotinin per ml plus 1 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (0.3-mm diameter) was added to the suspension, and cells were broken by vigorous vortexing for 5 min at 4°C. The beads and cell debris were removed by centrifugation at 13,000 × *g*. This crude extract was split, and part was treated with Triton X-100 (1%) prior to centrifugation at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in a volume of lysis buffer equal to that of the supernatant, and both were analyzed by SDS-PAGE on an 8% gel.

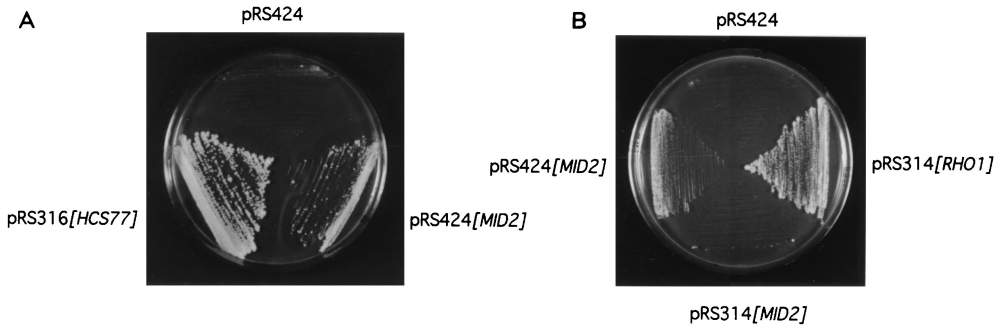

FIG. 1. Overexpression of *MID2* suppresses the cell lysis defect of an *hcs77*Δ *wsc2*Δ mutant and a conditional *rho1* mutant. (A) Transformants of yeast strain DL2230 (*hcs77*Δ *wsc2*Δ) harboring episomal vector (pRS424), pRS424[*MID2*], or centromeric plasmid pRS316[*HCS77*] were streaked onto a YEPD plate and incubated at 38°C for 3 days. (B) Transformants of yeast strain DL1235 (*rho1-5*) harboring episomal plasmid pRS424 or pRS424[*MID2*] or centromeric plasmid pRS314[RHO1] or pRS314[MID2] were streaked onto a YEPD plate and incubated at 37°C for 3 days.

RESULTS

The *HCS77* gene encodes a predicted type 1 membrane protein (9). Its most prominent features include a single transmembrane domain near its C terminus, a long Ser/Thr-rich region (ca. 145 residues), and a potential Cys-zinc finger near its N terminus. Loss of *HCS77* function results in a cell lysis defect during growth at 37 to 39°C (9, 16, 38), depending on the genetic background (unpublished results). This defect is accompanied by a deficiency in the ability to activate the Mpk1 MAP kinase in response to mild thermal stress (9). These findings, together with the localization of Hcs77 to the cell periphery (38), support a model in which Hcs77 functions as a sensor for cell integrity signaling (9, 16, 38).

Isolation of *MID2* as a dosage suppressor of the *hcs77*Δ defect. In the course of screening a centromeric library constructed to isolate the gene encoding a mutational suppressor of the *hcs77*Δ growth defect, we isolated a plasmid that contains the wild-type *MID2* gene. An *hcs77*Δ strain (DL1987) was transformed with a genomic yeast library and screened for suppression of its cell lysis defect at 39°C. We isolated from this screen a single plasmid (pRM5.2) which contains a region of chromosome 12 with three complete ORFs. One of these is *MID2* (YLR332W); the others were *RPS31* and an uncharacterized ORF, designated YLR331C. Deletion analysis of this plasmid revealed that *MID2* (for mating-induced death) was the gene responsible for suppression of *hcs77*Δ (data not shown). A *MID2* isolate from wild-type cells was able to suppress *hcs77*Δ when cloned into a centromeric vector. Moreover, DNA sequence analysis of the plasmid-borne *MID2* revealed no differences from the same region of DNA isolated by PCR from the isogenic wild-type strain, indicating that *MID2* acts as a dosage suppressor of the *hcs77*Δ defect, even when expressed from a low-copy-number plasmid. This suppression was more easily observed in an *hcs77*Δ *wsc2*Δ double mutant (Fig. 1A), which has a growth defect slightly more severe than that of the strain with *hcs77*Δ alone (38).

Because Hcs77 acts through the cell integrity signaling pathway, we examined the ability of *MID2* overexpression to suppress other mutant defects in signaling components thought to be under the control of Hcs77. Figure 1B shows that overexpression of *MID2* from a multicopy vector suppresses the growth defect of the *rho1-5* mutant (DL1235), which lyses at the restrictive temperature (19). This suppression was allele specific, because *MID2* failed to suppress the growth defects of *rho1-3* and *rho1-4* mutants (not shown), indicating that *MID2* overexpression does not bypass the requirement for *RHO1*. Strains with mutations in other genes that function downstream of *RHO1* (e.g., *PKC1*, *BCK1*, and *MPK1*) were not detectably suppressed by *MID2* overexpression.

***MID2* and *HSC77* act in parallel to regulate cell integrity signaling.** Three possibilities exist for the organization of a pathway in which both Hcs77 and Mid2 function. Mid2 might be under the control of Hcs77 such that overexpression of Mid2 drives pathway activation sufficiently to allow survival of thermal stress in the absence of signal from Hcs77. Alternatively, these proteins might act in parallel to provide a partially redundant function in the regulation of cell integrity signaling. Finally, Mid2 might positively regulate the expression of the *HCS77*-related genes *WSC2*, *WSC3*, and *WSC4*. However, steady-state mRNA levels from these genes were found to be unaffected by either overexpression or deletion of *MID2* (data not shown), ruling out this last possibility.

To distinguish between the two remaining possibilities, we first tested for suppression of the *mid2*Δ defect by *HCS77* overexpression. The *MID2* gene was isolated initially for its null mutant phenotype, which is failure to survive mating pheromone treatment (30). Treatment of a *MATa mid2*Δ mutant with α-factor resulted in 90% cell death after 5 h (Fig. 2).

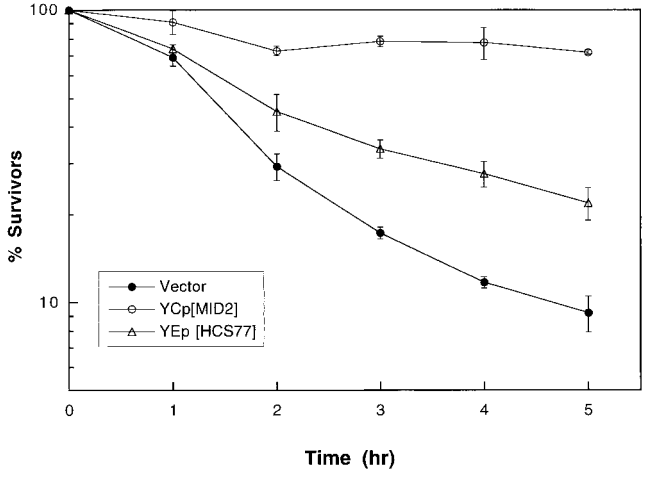

FIG. 2. Overexpression of *HCS77* suppresses the pheromone-induced death of a *mid2*Δ mutant. Yeast strain DL2277 (*MAT***a** *mid2*Δ) transformed with centromeric vector pRS314, pRS314[*MID2*], or episomal plasmid pRS424 [*HCS77*] was grown in SD with limiting calcium (100 μM $CaCl_2$) at 30°C to an A_{600} of 0.5 to 1.0. Mating pheromone (α-factor; 8 μg/ml) was added to the cultures, and samples were tested hourly for viability by plating onto YEPD. Plates were scored after 2 days at room temperature.

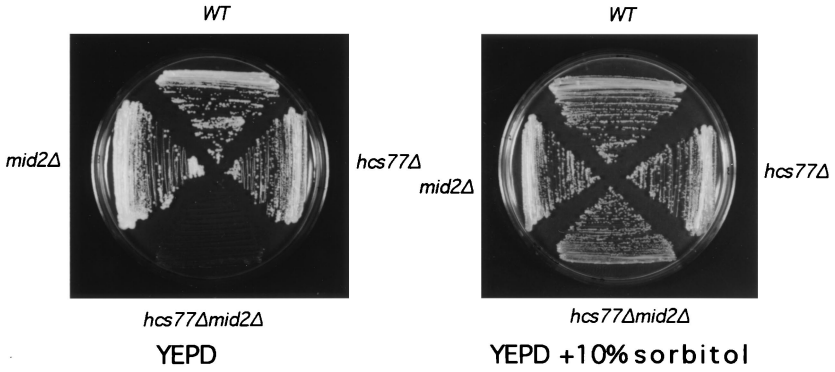

FIG. 3. A *mid2*Δ *hcs77*Δ mutant displays a severe cell lysis defect. Haploid yeast strains 1783 (wild type [WT]), DL2277 (*mid2*Δ), DL1985 (*hcs77*Δ), and DL2282 (*mid2*Δ *hcs77*Δ) were streaked onto YEPD and YEPD supplemented with 10% sorbitol for osmotic support and then incubated at room temperature for 2 days.

Overexpression of either *HCS77* (Fig. 2) or *RHO1* (not shown) from episomal plasmids partially suppressed this defect. The absence of an epistatic relationship, demonstrated by the reciprocal suppression of *hcs77*Δ by *MID2* and *mid2*Δ by *HCS77*, suggests that these genes function in parallel.

The possibility that Mid2 and Hcs77 share a redundant function is also supported by structural similarities between these proteins. The Mid2 polypeptide, like Hcs77, is predicted to be a type 1 membrane protein with its transmembrane region near the C terminus. Additionally, it possesses a long Ser/Thr-rich region (ca. 185 residues) that is N terminal to the transmembrane domain. These features, together with the ability of *MID2* to suppress *hcs77*Δ, suggests that these proteins might have overlapping functions, although Hcs77 and Mid2 show no sequence similarity outside these generally related regions.

One prediction of a model in which Mid2 and Hcs77 share a function is that the combined defect of mutations in both genes should be more severe than either defect alone. Therefore, we tested the *mid2*Δ and *hcs77*Δ mutations for additivity of growth defects. Either mutation alone did not result in a growth defect at temperatures as high as 37°C (not shown). In contrast, the double mutant was inviable even at room temperature (Fig. 3). This growth defect was suppressed by the addition of 10% sorbitol to the growth medium for osmotic support at temperatures as high as 34°C. Removal of osmotic support resulted in immediate cell lysis, as judged microscopically by a high frequency of nonrefractile ghosts. The behavior of the *mid2*Δ *hcs77*Δ mutant is characteristic of other mutants in the cell integrity signaling pathway.

Mid2 and Hcs77 are plasma membrane-associated proteins. Another prediction of a model in which Mid2 and Hcs77 function as sensors for cell integrity signaling is that they should localize to the plasma membrane. Hcs77 has been shown by fluorescence microscopy to be distributed uniformly around the cell periphery (38). To examine the intracellular localization of Mid2, we first fused GFP to the C terminus of Mid2. This fusion protein was fully functional, as judged by its ability to complement the pheromone sensitivity of the *mid2*Δ mutant when expressed from a centromeric plasmid (not shown). Fluorescence microscopy of cells expressing the Mid2-GFP fusion from the same plasmid revealed that this protein localizes uniformly around the cell periphery (Fig. 4A). By contrast, GFP alone, when expressed in yeast, is cytoplasmic (38). No fluorescent signal was detected from control cells (not shown). Treatment of cells with mating pheromone for up to 6 h did not appear to alter the localization of Mid2-GFP (not shown).

To examine the intracellular localization of Mid2 and Hcs77 biochemically, we fused the 3xHA epitope to their C termini. These fusions were fully functional, as judged by their ability to complement the defects associated with their deletion mutations when expressed from centromeric plasmids (not shown). Mid2-HA and Hcs77-HA were overexpressed from multicopy plasmids for the purpose of immunodetection. Both proteins migrated on SDS-PAGE as diffuse bands of much greater apparent molecular mass than those predicted by their amino acid sequences, suggestive of modification (see below). Figure 4B and C show that both Mid2-HA and Hcs77-HA fractionated completely with the pellet from a 100,000 × *g* centrifugation in the absence of detergent. In contrast, both proteins were solubilized in the presence of the nonionic detergent

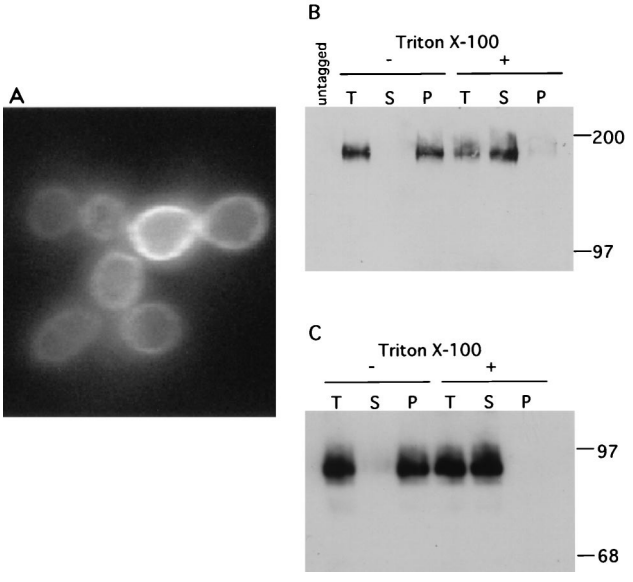

FIG. 4. Mid2 and Hcs77 localize to the plasma membrane. (A) Mid2-GFP, expressed in yeast strain DL2277 from a centromeric plasmid (pRS315[*MID2*-GFP]), was visualized with a fluorescein isothiocyanate filter. (B and C). Mid2-HA (B) and Hcs77-HA (C) were expressed from an episomal vector (YEp352) in yeast strain 1788. Crude extracts were split and subjected to a 100,000 × *g* centrifugation with or without 1% Triton X-100. Samples containing protein from the total extract (T; 46 μg of the Mid2-HA extract and 25 μg of the Hcs77-HA extract), supernatant (S), and pellet (P) were subjected to SDS-PAGE followed by immunoblot analysis. The control (untagged) lane in panel B contains total extract from cells of strain 1788 without an epitope-tagged protein. Sizes are indicated in kilodaltons.

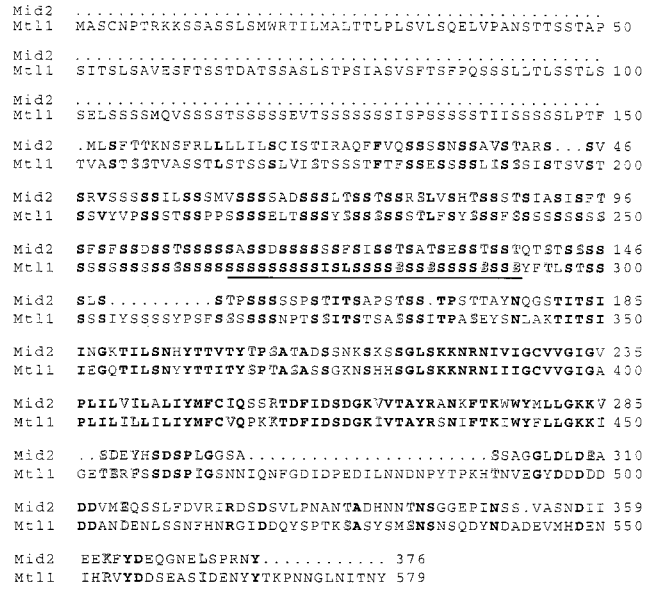

FIG. 5. Sequence alignment of Mid2 and Mtl1. Identical residues are in bold, similar residues are in shadow type. The underline indicates a 28-residue insertion in the predicted Mtl1 sequence from the EG123 background relative to the S288C sequence available through the yeast genome database.

Triton X-100 (1%), indicating that they are associated with a membrane. Taken together with the peripheral localization observed for Hcs77-GFP and Mid2-GFP, these results indicate that both proteins are associated with the plasma membrane. This finding, combined with the strongly additive growth defect of the *mid2*Δ *hcs77*Δ mutant and the reciprocal suppression results described above, indicates that these genes provide an essential but redundant function in the maintenance of cell integrity during vegetative growth.

Mid2 has a homolog, Mtl1. A search of the yeast genome database revealed that *MID2* has a homolog on chromosome 7. This uncharacterized ORF (YGR023) shows 50% amino acid sequence identity with Mid2 (Fig. 5). The gene was therefore designated *MTL1* (for *MID2*-like). We constructed a genomic deletion of *MTL1* (see Materials and Methods). An *mtl1*Δ mutant displayed no growth defect at temperatures up to 39°C, nor was it sensitive to pheromone-induced death (data not shown). However, a *mid2*Δ *mtl1*Δ mutant displayed an osmotic-remedial lysis defect when grown at 39°C (Fig. 6A), as evidenced by a high frequency of nonrefractile ghosts in the absence of osmotic support at the restrictive temperature. Overexpression of *HCS77* weakly suppressed this growth defect (Fig. 6B), further supporting a model in which these genes share a common function during vegetative growth. Loss of *MTL1* did not enhance the sensitivity of a *mid2*Δ mutant to pheromone-induced killing, nor did it confer pheromone sensitivity to an *hcs77*Δ mutant (not shown). However, overexpression of *MTL1* partially suppressed the pheromone sensitivity of *mid2*Δ. In contrast to *mid2*Δ, the *mtl1*Δ mutation did not exacerbate the cell lysis defect of an *hcs77*Δ mutant, nor did overexpression of *MTL1* suppress *hcs77*Δ. These results, taken in the aggregate, indicate that *MID2* plays a more important role in both vegetative growth and pheromone sensitivity than does *MTL1*.

Mild thermal stress is a condition known to activate the Mpk1 MAP kinase in wild-type cells (18). To examine the role of Mid2 and Mtl1 in cell integrity signaling during vegetative growth, we compared the activities of Mpk1 in wild-type and mutant strains in response to shift from growth at room temperature to 39°C for 1 h. Mpk1 was strongly activated by this treatment in wild-type cells (Fig. 6C; compare lanes 1 and 2). Temperature-induced activation of this protein kinase in an assay with myelin basic protein (MBP) as the substrate was somewhat diminished in both a *mid2*Δ mutant and an *mtl1*Δ mutant (compare lanes 4 and 5 and lanes 6 and 7, respectively). Mpk1 activation was severely impaired in the *mid2*Δ *mtl1*Δ mutant but not completely eliminated (compare lanes 8 and 9), consistent with the weak cell lysis defect of the double mutant. It should be noted that the double mutant survived this treatment for the duration of the experiment (data not shown). An *hcs77*Δ mutant, which is also markedly impaired for Mpk1 activation, also retains some ability to stimulate this kinase. Because an *hcs77*Δ *mid2*Δ mutant is inviable in the absence of osmotic support, and high osmolarity prevents activation of Mpk1 in response to thermal stress (18), we were unable to examine Mpk1 activation in this mutant.

***MID2* is required for cell integrity signaling in response to mating pheromone-induced morphogenesis.** Cell integrity signaling is activated by pheromone treatment at a time that is coincident with the onset of morphogenesis (8). Moreover, like *mid2* mutants, mutants that fail to activate cell integrity signaling are killed by pheromone treatment (8). Therefore, we examined the ability of a *mid2*Δ mutant to activate cell integrity signaling in response to treatment with mating pheromone. Cells that are synchronized at Start display a strong activation of the Mpk1 MAP kinase in response to α-factor treatment, which peaks approximately 1 h after exposure to the pheromone (1, 8) (Fig. 7). An isogenic *mid2*Δ mutant was greatly impaired in this activation. It should be noted that the *mid2*Δ mutant survived pheromone treatment for the duration of this experiment (not shown). Interestingly, the pheromone-induced death of the *mid2*Δ mutant was not suppressed by osmotic support (1 M sorbitol), in contrast to pheromone-induced death of an *mpk1*Δ mutant (8). This finding suggests that Mid2 may serve an additional function beyond regulation of cell integrity signaling.

Hcs77 and Mid2 are highly glycosylated proteins. Hsc77-HA is predicted to migrate on SDS-PAGE with a molecular mass of approximately 43 kDa. Instead, it migrated as a diffuse band with apparent molecular mass of 85 to 90 kDa, suggesting that Hcs77 is a highly glycosylated protein (Fig. 4B). This protein possesses two potential N-glycosylation sites (AsnSer/Thr) at positions 4 and 65, as well as many potential O-glycosylation sites in the Ser/Thr-rich region. To examine the glycosylation state of this protein, we first eliminated the potential N-glycosylation sites by mutating the acceptor Asn residues to Gln. Figure 8A shows that neither of the single mutants nor the double mutant had an altered mobility, indicating that Hcs77 is not N-glycosylated. Additionally, these mutants were fully functional for complementation of an *hcs77*Δ mutant (not shown).

The Mid2-HA protein behaved similarly to Hcs77-HA, having a predicted molecular mass of approximately 45 kDa, but migrating on SDS-PAGE with a much greater apparent molecular mass (160 to 180 kDa [Fig. 4C]). To examine these proteins for O-glycosylation, we immunoprecipitated them from cell extracts and subjected the immunocomplexes to α-mannosidase treatment. This treatment hydrolyzes the α-linkages between residues of O-linked mannose chains. α-Mannosidase can also hydrolyze mannosyl residues present at the termini of N-linked GlcNAc chains. Figure 8B shows that mannosidase treatment reduced Mid2-HA to a species of apparent molecular mass of approximately 45 kDa and re-

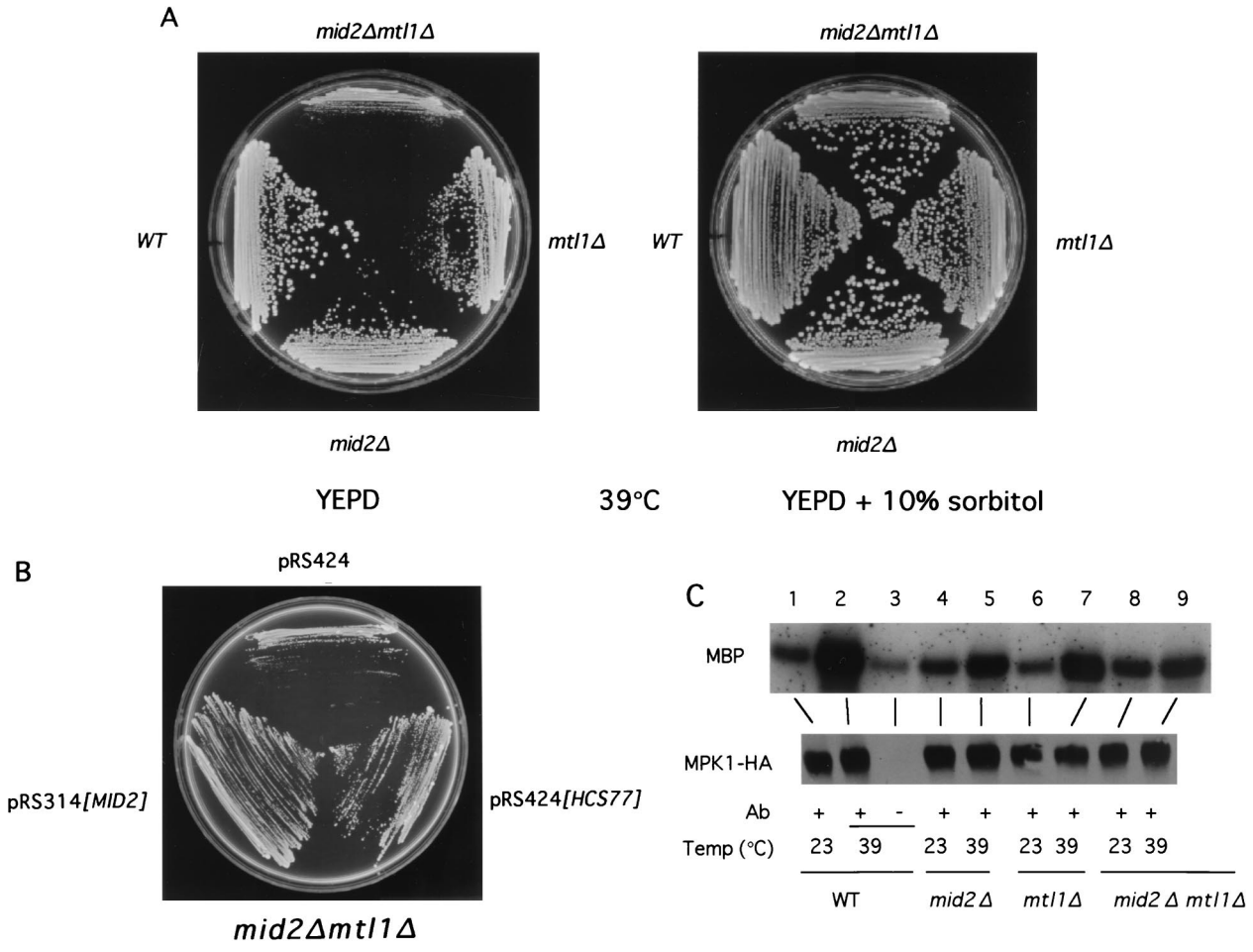

FIG. 6. A *mid2*Δ *mtl1*Δ mutant displays an osmotic-remedial cell lysis defect and is deficient in activating the Mpk1 MAP kinase. (A) Diploid yeast strains 1788 (wild type [WT]), DL2394, DL2395, and DL2396 were streaked onto YEPD or YEPD supplemented with 10% sorbitol and incubated at 39°C for 3 days. (B) Transformants of a *mid2*Δ *mtl1*Δ mutant (DL2396) harboring episomal plasmid pRS424, pRS424[*HCS77*], or centromeric plasmid pRS314[*MID2*] were streaked onto a YEPD plate and incubated at 39°C for 3 days. (C) The same yeast strains as in panel, A but transformed with YEp351[*MPK1*-HA], were cultured to mid-log phase at 23°C and shifted to 39°C for 1 h. Mpk1-HA was immunoprecipitated from extracts with monoclonal antibody (Ab) 12CA5, and the immunoprecipitates were subjected to protein kinase assays using MBP as the substrate. The lower panel displays an immunoblot of the Mpk1-HA immunoprecipitates.

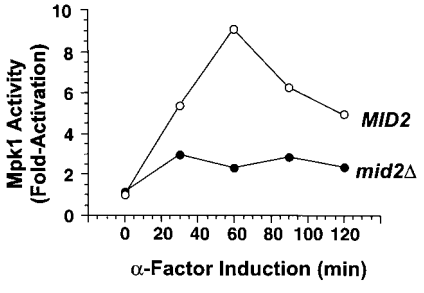

FIG. 7. Mid2 is required for pheromone-induced activation of Mpk1. Cultures of a *cdc28-13 mid2*Δ strain (DL2435) and an isogenic *MID2* strain (DL2393), both expressing Mpk1-HA from episomal plasmid pNC507 (1), were arrested in G_1 at 37°C and treated with mating pheromone (50 nM α-factor; $t = 0$). Mpk1-HA was immunoprecipitated from extracts of cultures taken at the indicated time points. Immunocomplex protein kinase assays were conducted with MBP as the substrate. Values represent ^{32}P-labeled MBP quantified by phosphorimager at each time point relative to that for the *MID2* extract assay at $t = 0$. Each value is the average of two independent experiments. Immunoblots of Mpk1-HA indicated that the levels of this protein did not change during the course of the experiment (not shown).

duced Hcs77-HA to a species of approximately 40 to 42 kDa. The observed values are nearly identical to the predicted masses of the unmodified proteins, confirming that Hcs77 (and strongly suggesting that Mid2) is specifically O-mannosylated.

DISCUSSION

***MID2* and *HCS77* act in parallel to stimulate cell integrity signaling.** The *HCS77* gene is thought to encode a cell surface sensor that detects cell wall weakness during vegetative growth. We isolated *MID2* as a low-copy-number dosage suppressor of the growth defect of an *hcs77*Δ mutant at 39°C. Several additional observations support a model in which Hcs77 and Mid2 are redundant sensors for cell integrity signaling during growth. First, an *hcs77*Δ *mid2*Δ double mutant displayed an osmotic remedial cell lysis defect that was much more severe than that of an *hcs77*Δ mutant, failing to grow at any temperature in the absence of osmotic support. Second, although loss of *MID2* function alone does not result in a growth defect (30), deletion of the *MTL1* gene, which encodes a Mid2-like protein (see below), in a *mid2*Δ mutant resulted in an osmotic remedial cell lysis defect at 39°C that was suppressed by overexpression of *HCS77*. Third, the ability to activate cell integrity signaling

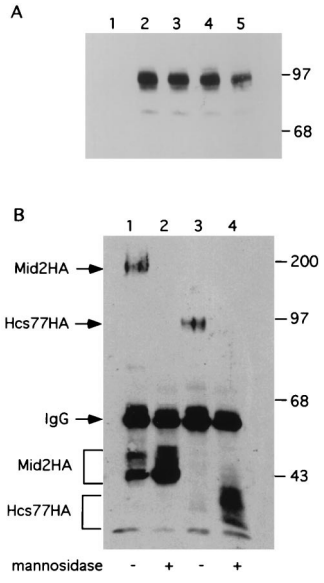

FIG. 8. Mid2 and Hcs77 are O-mannosylated. (A) Extracts (50 μg of protein) of yeast strain 1788 expressing Hcs77 (lane 1), Hcs77-HA (lane 2), $Hcs77^{N4Q}$-HA (lane 3), $Hcs77^{N65Q}$-HA (lane 4), or $Hcs77^{N4Q,N65Q}$-HA (lane 5) from YEp352 were subjected to SDS-PAGE followed by immunoblot analysis. (B) Mid2-HA (lanes 1 and 2) and Hcs77-HA (lanes 3 and 4) were immunoprecipitated from extracts (100 μg of protein) of yeast strain 1788. Immunoprecipitates were either treated with α-mannosidase at 37°C for 3 h (lanes 2 and 4) or mock treated with buffer only under the same conditions (lanes 1 and 3). Immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis. Sizes are indicated in kilodaltons. IgG, immunoglobulin G.

at elevated growth temperature, as measured by stimulation of the Mpk1 MAP kinase, was partially impaired by deletion of *HCS77*, *MID2*, or *MTL1*. Fourth, the structural similarity between Hcs77 and Mid2, together with their intracellular colocalization, also suggests redundancy in function. Specifically, they are both uniformly distributed through the plasma membrane, presumably by virtue of their single membrane-spanning regions. Additionally, they were both shown to be highly O-mannosylated through long Ser/Thr-rich regions that are predicted to reside extracellularly.

Overexpression of either *RHO1* or *PKC1* suppresses the growth defect of *hcs77*Δ cells (9, 38), suggesting that these components of the cell integrity pathway function downstream of *HCS77*. Similarly, we found that overexpression of *RHO1* also suppresses a *mid2*Δ mutant. However, overexpression of *MID2* was capable of suppressing the growth defect of one *rho1* mutant (*rho1-5*). We interpret this allele-specific suppression to indicate that *MID2* is not capable of bypassing the requirement for *RHO1* (either by acting downstream of or parallel to this G protein) but instead suppresses the *rho1-5* defect by acting upstream of this allele. Therefore, we propose that both Hcs77 and Mid2 contribute to Rho1 activation.

***MID2* is required for cell integrity signaling in response to mating pheromone treatment.** The *MID2* gene was isolated initially because its loss of function causes cell death in response to treatment with mating pheromone (30). Mutants in *MID2* arrest growth in G_1 and undergo morphogenesis in response to pheromone but fail to recover from this arrest. Activation of cell integrity signaling is a late response to pheromone treatment that, like *MID2*, is essential for survival of pheromone-induced morphogenesis (8). In addition to the role that Mid2 plays in signaling cell wall stress during vegetative growth, we demonstrated that it is also critical for cell integrity signaling during pheromone-induced morphogenesis. Interestingly, in contrast to the behavior of an *mpk1*Δ mutant in response to pheromone (8), the pheromone sensitivity of a *mid2*Δ mutant is not suppressed by the addition of osmotic support to the medium (to prevent cell lysis), suggesting that Mid2 may have an additional function in the survival of pheromone treatment. Intriguingly, *MID2* was also isolated as a dosage suppressor of a strain with a mutation of *MPT5* (37), which has been proposed to act through the Cdc28 cyclin-dependent kinase to stimulate passage through G_1 in pheromone-arrested cells (2). Therefore, Mid2 may have a cell cycle function that is independent of its role in cell integrity signaling. In contrast to Mid2, Hcs77 appears to be dedicated to signaling cell wall perturbation during vegetative growth and thermal stress, because a null mutant did not display sensitivity to killing by mating pheromone (32a).

Hcs77 and Mid2 as sensors of cell wall stress. The predicted topography of these plasma membrane proteins, based on the positions of their signal sequences, indicates that they have short C-terminal cytoplasmic domains and longer extracellular domains that are very rich in seryl and threonyl residues. These domains are separated by a single transmembrane sequence. The extracellular domains are highly O-mannosylated. In yeast, this modification consists of several (four or five) mannosyl residues in α-1,2 and 1,3 linkages (36). When many such modifications are present in a stretch of seryl/threonyl residues, as appears to be the case for both Hcs77 and Mid2, they induce the polypeptide to adopt a stiff and extended conformation (17). Therefore, Hcs77 and Mid2 may function as molecular probes that span the periplasmic space to interact directly with the cell wall. If these proteins are glycosylated throughout their Ser/Thr-rich regions, the calculated extents of Mid2 (ca. 470 Å) and Hcs77 (ca. 370 Å) are adequate to span the distance from the plasma membrane to the cell wall (ca. 200 to 300 Å).

Mid2 and Hcs77 are predicted to have overlapping intracellular targets. Paradoxically, however, their predicted intracellular domains are not similar at the level of primary sequence. They may interact with different complexes that share common signaling components, or they may be linked to the cell integrity signaling apparatus through different adaptor molecules.

The Mid2 homolog, Mtl1. We isolated a gene predicted to encode a polypeptide showing 50% amino acid sequence identity with Mid2. Loss of *MTL1* function did not result in any overt defects, either during vegetative growth or in response to mating pheromone, nor did it exacerbate either the pheromone sensitivity of a *mid2*Δ mutant or the cell lysis defect of an *hcs77*Δ mutant. However, a *mid2*Δ *mtl1*Δ mutant displayed a weak cell lysis defect when cultivated at high temperature and was more impaired for thermal activation of the Mpk1 MAP kinase than was a *mid2*Δ mutant. Therefore, *MTL1* appears to play a minor role in cell integrity signaling during vegetative growth.

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