EUKARYOTIC CELL, Aug. 2005, p. 1364–1374 Vol. 4, No. 8
1535-9778/05/$08.00+0 doi:10.1128/EC.4.8.1364–1374.2005

Yapsins Are a Family of Aspartyl Proteases Required for Cell Wall Integrity in *Saccharomyces cerevisiae*

Damian J. Krysan,[1]* Elizabeth L. Ting,[2] Claudia Abeijon,[2] Lee Kroos,[3] and Robert S. Fuller[4]*

Department of Pediatrics and Communicable Diseases, Division of Pediatric Infectious Disease, University of Michigan Hospitals,[1] and Department of Biological Chemistry, University of Michigan Medical School,[4] Ann Arbor, Michigan 48109; Department of Molecular and Cell Biology, Goldman School of Dental Medicine, Boston University, Boston, Massachusetts 02118[2]; and Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824[3]

Received 6 May 2005/Accepted 9 June 2005

The yeast cell wall is a crucial extracellular organelle that protects the cell from lysis during environmental stress and morphogenesis. Here, we demonstrate that the yapsin family of five glycosylphosphatidylinositol-linked aspartyl proteases is required for cell wall integrity in *Saccharomyces cerevisiae*. Yapsin null mutants show hypersensitivity to cell wall perturbation, and both the *yps1*Δ*2*Δ mutant and the quintuple yapsin mutant (5*yps*Δ) undergo osmoremedial cell lysis at 37°C. The cell walls of both 5*yps*Δ and *yps1*Δ*2*Δ mutants have decreased amounts of 1,3- and 1,6-β-glucan. Although there is decreased incorporation of both 1,3- and 1,6-β-glucan in the 5*yps*Δ mutant in vivo, in vitro specific activity of both 1,3- and 1,6-β-glucan synthesis is similar to wild type, indicating that the yapsins affect processes downstream of glucan synthesis and that the yapsins may be involved in the incorporation or retention of cell wall glucan. Presumably as a response to the significant alterations in cell wall composition, the cell wall integrity mitogen-activated kinase signaling cascade (*PKC1-MPK* pathway) is basally active in 5*yps*Δ. *YPS1* expression is induced during cell wall stress and remodeling in a *PKC1-MPK1*-dependent manner, indicating that Yps1p is a direct, and important, output of the cell wall integrity response. The *Candida albicans* (*SAP9*) and *Candida glabrata* (*CgYPS1*) homologues of *YPS1* complement the phenotypes of the *yps1*Δ mutant. Taken together, these data indicate that the yapsins play an important role in glucan homeostasis in *S. cerevisiae* and that yapsin homologues may play a similar role in the pathogenic yeasts *C. albicans* and *C. glabrata*.

The yapsins are a family of five glycosylphosphatidylinositol (GPI)-linked aspartyl proteases (*YPS1* to *-3*, *-6*, and *-7*) in *Saccharomyces cerevisiae* that have homologues in other fungi such as *Candida albicans* (43), *Candida glabrata* (16), and *Aspergillus oryzae* (36). The founding members of this family, *YPS1* and *-2* (17, 32), were identified as suppressors of null mutations in *KEX2*, a trans-Golgi network-localized serine protease that processes secretory proteins such as pro-α-factor and the exoglucanase Exg1p (3, 53). The remaining three yapsins were subsequently identified by sequence homology following completion of the *S. cerevisiae* genome sequence (47). Like Kex2p, three of the yapsins (Yps1p, -2p, and -3p) cleave proteins and peptides C terminal to basic residues, both in vivo and in vitro (8, 33). Although Kex2p is exquisitely selective for Lys-Arg pairs (53), the yapsins cleave C terminal to monobasic sites containing either Lys or Arg (33, 48). In vivo, Yps1p cleaves human mammalian prohormones such as ACTH and CCK (8) and both Yps1p and Yps2p display secretase-like activity toward β-amyloid precursor protein that is expressed in yeast (34, 66). To date, however, no yeast substrates have been identified; moreover, the physiologic function of the yapsins in *S. cerevisiae* has been unclear.

Previously reported work from this laboratory showed that the yapsin double null mutant *yps1*Δ*yps2*Δ has a growth defect at 37°C (32). Although this phenotype is not highly specific, it is, nonetheless, characteristic of strains with cell wall defects, and we hypothesized that the yapsins may process cell wall proteins involved in the maintenance of cell wall integrity. A possible role for the yapsins in cell wall function is also supported by the fact that *YPS1*, *YPS3*, and *YPS6* have been shown by genome-wide expression experiments to be induced during periods of cell wall stress and remodeling (7, 20, 21, 27). Supporting the notion that *YPS1* is induced during periods of cell wall stress, quantitative immunoblotting experiments have shown that Yps1p is increased 12-fold when cells are shifted from 24°C to 37°C (2). Similarly, Yps1p could not be detected by immunofluorescence at 24°C, but robust plasma membrane-localized fluorescence was evident at 37°C (58). Additionally, a recent genome-wide screen for mutants resistant to killer toxin, and, therefore, involved in 1,6-β-glucan-related processes, identified the *yps7*Δ mutant (51). Finally, a genome-wide screen for synthetically lethal double mutants identified *mpk1*Δ*yps7*Δ (62). *MPK1* encodes the terminal mitogen-activated protein kinase (MAPK) in the *PKC1-SLT2/MPK1* pathway, one of the most important signaling cascades for the maintenance of cell wall integrity (24). The synthetic lethal interaction between *mkp1*Δ and *yps7*Δ suggests that loss of *YPS7* function leads to activation of the cell wall integrity pathway as an essential compensatory response. Taken to-

* Corresponding author. Mailing address for Damian J. Krysan: Department of Pediatrics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642. Phone: (585) 275-5944. Fax: (585) 756-7780. E-mail: damian_krysan@urmac.rochester.edu. Mailing address for Robert S. Fuller: Department of Biological Chemistry, University of Michigan Medical School, 5413 Medical Science Building I, 1303 East Catherine Street, Ann Arbor, MI 48109. Phone: (734) 936-9764. Fax: (734) 763-7799. E-mail: bfuller@umich.edu.

TABLE 1. Yeast strains

Strain	Genotype	Source
CRY1 (W3031A)	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*	Laboratory collection
HKY20	CRY1 *yps1*Δ::*LEU2*	32
HKY21	CRY1 *yps2*Δ::*HIS3*	32
DKY5	CRY1 *yps3*Δ::*KANMX4*	This study
DKY7	CRY1 *yps6*Δ::*KANMX4*	This study
DKY9	CRY1 *yps6*Δ::*NATMX3*	This study
DKY11	CRY1 *yps7*Δ::*KANMX4*	This study
DKY13	CRY1 *yps7*Δ::*NATMX3*	This study
HKY24	CRY1 *yps1*Δ::*LEU2 yps2*Δ::*HIS3*	32
DKY17	CRY1 *yps1*Δ::*LEU2 yps3*Δ::*KANMX4*	This study
DKY19	CRY1 *yps1*Δ::*LEU2 yps6*Δ::*KANMX4*	This study
DKY21	CRY1 *yps1*Δ::*LEU2 yps7*Δ::*KANMX4*	This study
DKY23	CRY1 *yps2*Δ::*HIS3 yps3*Δ::*KANMX4*	This study
DKY25	CRY1 *yps2*Δ::*HIS3 yps6*Δ::*KANMX4*	This study
DKY27	CRY1 *yps2*Δ::*HIS3 yps7*Δ::*KANMX4*	This study
DKY29	CRY1 *yps3*Δ::*KANMX4 yps6*Δ::*NATMX3*	This study
DKY31	CRY1 *yps3*Δ::*KANMX4 yps7*Δ::*NATMX3*	This study
DKY33	CRY1 *yps6*Δ::*KANMX4 yps7*Δ::*NATMX3*	This study
DKY35	HKY24 *yps3*Δ::*KANMX4*	This study
DKY39 (5*yps*Δ)	DKY10 *yps6*Δ::*KANMX4 yps7*Δ::*KANMX4*	This study
DDY2080	*cbk1*::*KANMX4*	65
DKY41	*cbk1*::*KANMX4 yps1*::*LEU2*	This study
BY4741	*his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0*	Research Genetics
*mpk1*Δ	BY4741 *mpk1*Δ::*KANMX4*	Research Genetics
*rlm1*Δ	BY4741 *rlm1*Δ::*KANMX4*	Research Genetics
*crz1*Δ	BY4741 *crz1*Δ::*KANMX4*	Research Genetics
*fks1*Δ	BY4741 *fks1*Δ::*KANMX4*	Research Genetics
*smi1*Δ	BY4741 *smi1*Δ::*KANMX4*	Research Genetics
*chs3*Δ	BY4741 *chs3*Δ::*KANMX4*	Research Genetics
*mnn10*Δ	BY4741 *mnn10*Δ::*KANMX4*	Research Genetics

gether, these pieces of information suggested a role for the yapsins in cell wall processes, but prior to our study no dedicated investigation of such a role had been reported.

The yeast cell wall is a vital extracellular organelle that determines cell shape, protects the cell from lysis, and adapts to a variety of morphogenetic changes during the yeast life cycle (49). Because of the last requirement, the cell wall is a dynamic structure that undergoes significant remodeling of both its molecular composition and structural organization in response to a variety of internal and external stimuli (31, 59). The fact that all five of the MAP kinase cascades in *S. cerevisiae* affect the expression of genes with roles in cell wall physiology underscores the importance of the integrity of the cell wall to yeast cell biology (22). Examples of cell wall stress-activated genes include those for glucan synthase (*FKS2*), chitin synthase (*CHS3*), and a variety of cell wall mannoproteins (e.g., *CWP1*, *ECM11*, and *PRH1* [28]). Of the many genes whose expression is affected by cell wall stress, relatively few encode proteins with biochemical activities that have been demonstrated experimentally or deduced by homology (49). Here, we demonstrate that the yapsin family of proteases is required for the maintenance of yeast cell wall integrity and appears to be involved principally in cell wall glucan homeostasis. We also show that Yps1p, in particular, is part of the transcriptional response to cell wall stress and required during severe cell wall stress in *S. cerevisiae*. Finally, we provide genetic evidence suggesting that the yapsin homologues in the human pathogens *C. albicans* and *C. glabrata* may also be involved in cell wall homeostasis.

MATERIALS AND METHODS

Yeast strains and growth conditions. Strains were constructed in the W303 background using standard procedures in yeast genetics (5) or obtained from the S288C deletion collection (Research Genetics) (Table 1). *NATMX4*-marked mutants were constructed from *KANMX4* strains using the method of Tong et al. (62). Yeast cells were grown using rich medium (YPD, 1% yeast extract-2% peptone-2% glucose) or synthetic dropout medium prepared in the standard manner (5). SYPD refers to YPD plates or liquid medium containing 1.0 M sorbitol. For growth on agar, YPD supplemented with 0.004% adenine was used. Calcofluor white, Congo Red, and caffeine (Sigma, St. Louis, MO) were used to prepare drug-supplemented YPD. Caspofungin acetate (Merck) was purchased from the University of Michigan Hospital Pharmacy, and YPD-caspofungin plates were prepared as described previously (38). Drug sensitivity was assayed by growth at 30°C for 2 to 3 days unless otherwise noted.

Plasmids. Previously published plasmids were obtained from the authors (Table 2).

(i) pr*YPS1-lacZ*. The *YPS1* promoter-driven *lacZ* reporter fusion was constructed by ligating a 665-bp BglII-SalI fragment of pRS4*YAP3*, containing sequence 5′ to *YPS1* as well as the ATG, to BamHI-SalI-digested YEp375R (45).

(ii) pRS426-*GPD-SAP9*. pRS426-*GPD-SAP9* was constructed by PCR amplifying a 1,711-bp fragment containing the *SAP9* gene from pSAP9 (generously provided by M. Monod [43]) with primers containing a 5′ BamHI site and a 3′ XhoI site. The fragment was digested with BamHI and XhoI and ligated into pRS426-*GPD* digested with the same restriction enzymes.

Cloning of *CgYPS1*. pRS316-*CgYPS1* was constructed by PCR amplifying a 2,314-bp fragment containing open reading frame CAGLOM04191g (*C. glabrata* genome website: http://cbi.labri.fr/Genolevures/elt/CAGL) and 500 bp of 5′-flanking sequence from genomic DNA from a clinical isolate of *C. glabrata* (kindly provided by D. Newton, University of Michigan) with primers containing 40 bp of 5′ and 3′ sequence homologous to the multicloning region of pRS416. pRS416 was linearized with SalI and XhoI and gel purified. The PCR product and the linearized vector were cotransformed into HKY20 (*yps1*Δ). Transformants were selected on synthetic dextrose medium minus uracil (SDC−URA), and 15 candidates containing the desired construct were identified by colony

TABLE 2. Plasmids

Plasmid	Description	Reference or source
pr*YPS1-lacZ*	665-bp 5′ promoter region of *YPS1* fused to *lacZ* in Yep375R	This study
pRS314-*YPS1*	pRS314 with *YPS1* expressed from native promoter	32
pRS314-*YPS2*	pRS314 with *YPS2* expressed from native promoter	32
pG5*YPS1*	pG5 with *YPS1* expressed from *TDH* promoter	34
pRS426-*GPD-SAP9*	1,711-bp fragment containing *SAP9* expressed from GPD promoter	This study
pRS416-*CgYPS1*	2,314-bp fragment containing *CgYPS1* and 500 bp 5′ to start codon in pRS416	This study
pDL922	Yep352-*MPK1*-HA(×3)	29
pXZ358	pRS414-P_{ADH}-*KSS1*-HA(×3)	K.-L. Liang

PCR. The 15 candidates were streaked onto SDC−URA along with HKY20 harboring the empty vector pRS416. After 2 days of growth, the strains were replica plated to YPD plus 50 mg/ml Congo Red. Twelve of 15 isolates grew under these conditions, while empty vector control did not. The sequence of one isolate of pRS416-*CgYPS1* was then confirmed.

Immunoblots. Strains harboring plasmids encoding Mpk1p-hemagglutinin (HA) (pDL922) or Kss1p-HA (pXZ358) were grown overnight to early log phase in SDC−URA at 30°C and harvested. The cells were resuspended in YPD and either heated at 37°C for 2 h (Mpk1p-HA) or treated with α-factor (0.125 μM in methanol; Sigma) for 15 min at 30°C (Kss1p-HA). Control cultures were maintained at 30°C for the same period or treated with an equal volume of methanol. Protein extracts were prepared as described previously (13), and equal amounts (15 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gel), transferred to nitrocellulose, and probed with anti-active MAPK (Promega) to detect either phospho-Mpk1p or phospho-Kss1p following the supplier's protocol. Comparable protein loading was confirmed by probing the stripped membranes (0.1 M glycine, pH 2.5) with 12CA5 (Roche) to detect the triple HA epitope. Blots were visualized using horseradish peroxidase-conjugated rabbit anti-donkey, secondary antibodies (Amersham), and the ECL-Plus Western blot detection kit (Amersham). Images were collected with a Typhoon Phosphorimager (Amersham) and processed using Adobe Photoshop software.

Cell wall analysis. To determine cell wall composition, yeast cells (25-ml cultures in YP-0.2% glucose or minimal medium) were grown at 23°C for seven to eight generations in the presence of 25 μCi of [U-^{14}C]glucose (310 mCi/mmol; New England Nuclear) and harvested in log phase. For the pulse-labeling experiments, 25 ml of logarithmically growing cells was preincubated for 30 min at 23°C followed by 5-min incubations with 100 μCi of [U-^{14}C]glucose (310 mCi/mmol; New England Nuclear) and harvesting. Cell walls were isolated from 100 to 300 mg of cells, and cell wall polysaccharides were fractionated and quantified as previously described (40).

1,3-β-Glucan synthase assay. In vitro 1,3-β-glucan synthase activity was measured according to a procedure adapted from a previously reported protocol (19). Yeast cells (50-ml culture) were grown to log phase (optical density at 600 nm [OD_{600}] of 0.8 to 1.0) in YPD at 30°C and harvested. Following glass bead lysis in 500 mM NaCl-1 mM EDTA-1 mM phenylmethylsulfonyl fluoride and centrifugation at 4,000 × *g*, the supernatant was removed and subjected to further centrifugation at 100,000 × *g* for 60 min at 4°C to give a membrane pellet. The pellet was resuspended in 50 mM Tris, pH 7.5, containing 66% (vol/vol) glycerol and stored at −80°C. Protein content was determined using the Bradford assay (Bio-Rad). Each reaction mixture contained 60 to 90 μg of membrane extract protein in a total reaction volume of 100 μl containing 80 mM Tris, pH 7.5, 20 μM GTP-γS, 6.6% (vol/vol) glycerol, 1 mM EDTA, 0.8% (wt/vol) bovine serum albumin (BSA), 2 mM NaF, and 2 mM UDP-[^{1}H]glucose carrier with UDP-[^{3}H]glucose (14,564 cpm/nM, 4 μCi/reaction). Reactions were conducted at 30°C for 60 min and were quenched by addition of 1 ml of ice-cold 90% methanol. The methanol-insoluble products were filtered through 25-mm glass fiber filters (GFA; Whatman), washed with ice-cold 90% methanol (2.5 ml) and ice-cold 90% ethanol (2.5 ml), and quantitated by liquid scintillation. Reactions were performed in duplicate using extracts from three separate clones for each strain.

1,6-β-Glucan synthase assay. In vitro 1,6-β-glucan synthase activity was measured as described previously (64). Briefly, membranes were prepared as described for the 1,3-β-glucan synthase assays (see above). The membrane pellet was resuspended in 50 mM Tris, pH 7.5, containing 33% glycerol, and 5 μg of membrane extract protein was used for each reaction in a total volume of 40 μl containing 2.5 mM UDP-glucose, 150 mM GTP, 1.3 mM EDTA, 0.75% (wt/vol) BSA, 4% (vol/vol) glycerol, and 100 mM morpholineethanesulfonic acid, pH 6.5. Reactions were conducted at 30°C, and 2-μl aliquots were removed at 15, 30, 60, and 120 min and directly spotted onto nitrocellulose membranes. The membranes were dried for 30 min and blocked with 5% (wt/vol) nonfat dry milk in PBS overnight at 4°C before being probed with rabbit anti-1,6-β-glucan antibodies (generous gift of Frans Klis [Amsterdam, The Netherlands]) at a dilution of 1:1,000 in PBS with 3% (wt/vol) BSA. The membranes were then probed with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham) at a dilution of 1:1,000 in PBS with 3% (wt/vol) BSA. Images were collected on a Typhoon Phosphorimager (Amersham) and quantitated using the histogram function of Adobe Photoshop software. The time course was used to confirm that the 1-h time point was in a linear range, and the amount of 1,6-β-glucan at 1 h was determined for the interstrain comparison. Each assay was done side by side with a wild-type strain, and the data represent the means of two independent experiments (performed in duplicate) using extracts from separate clones of each strain.

β-Galactosidase and AP assays. β-Galactosidase assays were performed as described previously (56) with cell extracts obtained by glass bead lysis. Specific activity is expressed as Miller units (nmoles/minute/mg of protein) at 37°C. Alkaline phosphatase (AP) activity was assayed using paranitrophenylphosphate as the chromogenic substrate according to a published procedure (42). Activity is expressed as OD_{420} absorbance normalized to the OD_{600} of the culture sample. All assays were performed in duplicate or triplicate, and the reported values represent the means of three independent experiments using extracts from separate clones of each strain.

RESULTS

Yapsin deletion mutants undergo osmoremedial cell lysis at 37°C. To investigate the role of the yapsins in cell wall integrity, we constructed a full set of single and double yapsin deletion mutants as well as selected multiple yapsin deletion mutants (Table 1). None of the single mutants showed growth defects at 37°C, and *yps1*Δ*yps2*Δ was the only double mutant that displayed temperature sensitivity (TS$^-$). The *yps1*Δ*yps2*Δ*yps3*Δ mutant was more severely affected than *yps1*Δ*yps2*Δ. Interestingly, the quintuple yapsin mutant (5*yps*Δ) was viable and showed no growth defect at 30°C (Fig. 1A). Consistent with the fact that only the *yps1*Δ*yps2*Δ double mutant exhibited TS$^-$, reintroduction to the 5*yps*Δ background of either *YPS1* or *YPS2* under endogenous promoters on yeast centromeric plasmids was sufficient to complement the TS$^-$ phenotype of the 5*yps*Δ mutant, suggesting that these two proteases may have partially overlapping functions (Fig. 1B).

Although mutations affecting a variety of cellular processes can cause TS$^-$ growth, mutations that disrupt cell wall integrity typically cause cells to lyse at elevated temperatures, a phenotype that can be suppressed by the addition of an osmotic support to the medium (42). Supplementing YPD medium with 1.0 M sorbitol restored growth of the TS$^-$ yapsin mutants to the level of wild-type cells at 37°C, implying that the yapsin

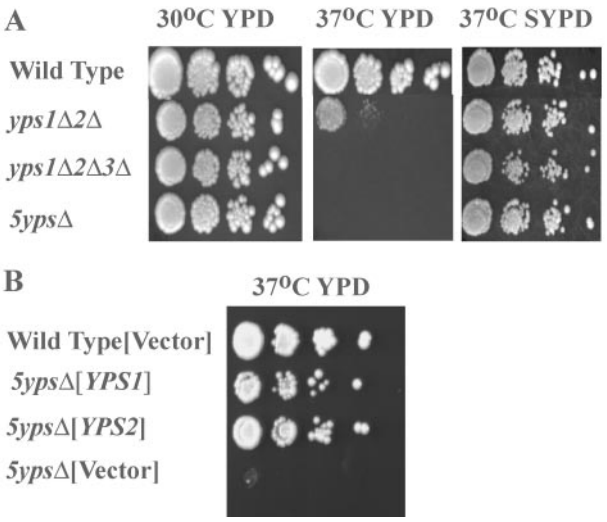

FIG. 1. Yapsin deletion mutants undergo osmoremedial cell lysis at 37°C. (A) Strains with multiple yapsin deletions show a profound growth defect on YPD at 37°C that is suppressed by the addition of 1.0 M sorbitol to the medium. (B) Reintroduction of either *YPS1* or *YPS2* (CEN plasmids under endogenous promoters) to the 5*yps*Δ background complements temperature sensitivity.

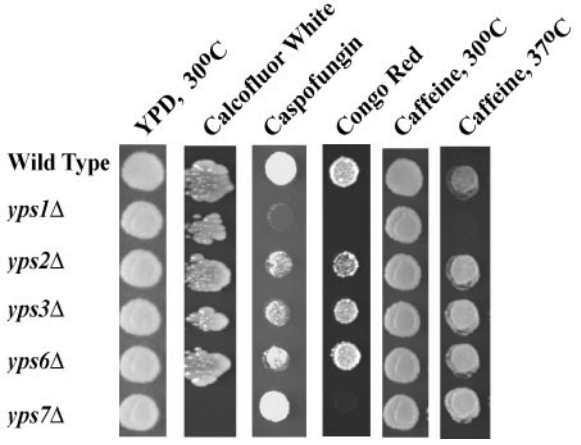

FIG. 2. Yapsin deletion mutants show differential susceptibility to cell wall-active drugs. Cells (5 μl of a 1-OD_{600} suspension) were pronged onto YPD plates supplemented with the indicated drug. Drug concentrations: calcofluor white, 20 μg/ml; caspofungin, 200 ng/ml; Congo Red, 200 μg/ml; caffeine, 6 mM. Plates were incubated at 30°C (unless otherwise indicated) for 3 to 5 days.

mutants undergo lysis at elevated temperature (Fig. 1A). Lysis of the yapsin mutants at elevated temperature was confirmed by measuring the release of AP, an intracellular enzyme, into the medium at 37°C (Table 3). The *yps1*Δ*yps2*Δ and 5*yps*Δ mutants both release AP at 37°C, and consistent with the plate assays, the AP activity released by the yapsin mutants at elevated temperature is decreased in cultures containing 1.0 M sorbitol. Taken together, these observations establish that the yapsins are involved in the maintenance of cell wall integrity and that loss of yapsin function results in cell lysis at elevated temperature.

Yapsin deletion mutants are differentially susceptible to cell wall-directed drugs. To characterize further the cell wall defects associated with loss of yapsin function, we examined the growth of the yapsin deletion mutants in the presence of four cell wall-disrupting drugs: calcofluor white, a dye that binds and disrupts chitin polymers (52); Congo Red, a dye that interferes with both chitin and β-glucan fibril formation (35); caffeine, a compound that induces cell wall stress, possibly by affecting TOR signaling (39); and caspofungin, an inhibitor of 1,3-β-glucan synthase (38). Not unexpectedly, the quintuple yapsin mutant, 5*yps*Δ, was exquisitely sensitive to all four agents (data not shown), while the single yapsin mutants displayed differential sensitivity to calcofluor white, Congo Red, caffeine, and caspofungin (Fig. 2). The *yps7*Δ strain was uniquely hypersensitive to calcofluor white, suggesting that this mutant may be dependent on chitin to reinforce the cell wall and, thereby, maintain cell wall integrity (52). In response to caffeine, none of the yapsin mutants showed growth defects at 30°C. The *yps1*Δ mutant was, however, sensitive to caffeine at 37°C. Although the cellular target of caffeine is unclear, it causes generalized cell wall stress, and the increased sensitivity of the *yps1*Δ mutant suggests that *YPS1* is required to maintain cell wall integrity under severe stress. The *yps1*Δ mutant was also sensitive to the 1,3-β-glucan synthase inhibitor caspofungin, while all other yapsin mutants behaved similarly to wild type. This suggests that *YPS1* may have an important role in 1,3-β-glucan homeostasis. Both the *yps1*Δ and *yps7*Δ mutants were hypersensitive to Congo Red, a dye that interferes with both 1,3-β-glucan and chitin fibril formation in the cell wall (35). These experiments indicate that the individual yapsins are not functionally redundant but instead appear to have distinguishable roles in cell wall biology.

Next, we tested the full set of 10 yapsin double mutants for

TABLE 3. Alkaline phosphatase activity[a] released into culture medium

Strain	25°C YPD	37°C YPD	37°C YPD plus 1 M sorbitol
Wild type	0.9 (0.1)[b]	1.9 (0.3)	2.0 (0.2)
*yps1*Δ*2*Δ	0.9 (0.5)	24.5 (1.8)	3.0 (0.1)
5*yps*Δ	3.0 (0.2)	517 (128)	17.6 (3.3)

[a] The alkaline phosphatase activity of culture medium was determined by measuring the hydrolysis of paranitrophenylphosphate and normalizing the absorbance at 420 nm to the optical density of the culture (42) to give an arbitrary absorbance unit/OD_{600}.

[b] Values in parentheses are the standard deviations of three independent experiments performed in duplicate.

TABLE 4. Synthetic genetic interactions of yapsin double mutants[a]

Parental strain	*yps1*Δ	*yps2*Δ	*yps3*Δ	*yps6*Δ	*yps7*Δ
*yps1*Δ	—	TS, CF, CR, CS, CW		CR, CS	CR, CS
*yps2*Δ	—	—			CW
*yps3*Δ	—	—	—		
*yps6*Δ	—	—	—	—	CR

[a] Table entries indicate decreased growth relative to both wild-type and parental strains under the tested conditions. Blank spaces indicate no synthetic interaction. "—" indicates redundant entry. Growth conditions: all incubations were on YPD with indicated supplements at 30°C unless noted otherwise. TS, 37°C; CF, caffeine; CR, Congo Red; CS, caspofungin; CW, calcofluor white.

TABLE 5. Cell wall composition of yapsin mutants[a]

Strain	1,3-β-Glucan	1,6-β-Glucan	Mannan	Chitin
WT	100 (2,331)[b]	100 (1,083)	100 (1,776)	100 (148)
*yps1*Δ*2*Δ	86	82	101	174
5*yps*Δ	57	69	97	202
5*yps*Δ[*YPS2*]	78	82	168	169
*fks1*Δ[c]	60	82	116	489

[a] All data are percentage of wild-type (WT) incorporation of [^{14}C]glucose label per mg of dry cell wall and are means of duplicate experiments except for *yps1*Δ*2*Δ, which is the mean of duplicates of a single experiment (standard deviation, <2% for all values).
[b] Label uptake in cpm/mg of dry cell wall is shown in parentheses for wild type.
[c] Data are from the work of Magnelli et al. (40) and are for comparison.

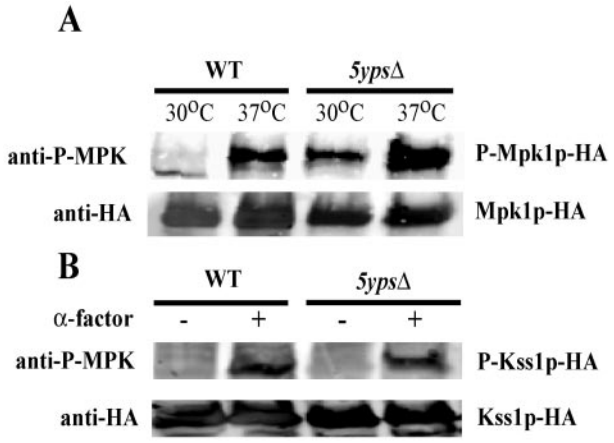

FIG. 3. *PKC1-MPK1* is basally activated in 5*yps*Δ. The phosphorylation of MAP kinases Mpk1p and Kss1p was assayed by immunoblot analysis of protein extracts from wild-type and 5*yps*Δ cells harboring *MPK1*-HA (pDL922) or *KSS1*-HA (pXZ358) using the phosphospecific antibody anti-active MAPK (Promega). Anti-HA (12CA5) was used to demonstrate equal protein loading. (A) Mpk1p is not phosphorylated at 30°C in wild type (WT) but is phosphorylated at 30°C in the 5*yps*Δ mutant. (B) Kss1p, the MAPK of the SVG pathway, is not phosphorylated at 30°C in either wild-type or 5*yps*Δ strains but is responsive to α-factor.

synthetic genetic interactions in the presence of the cell wall-perturbing drugs (Table 4). Synthetic effects were observed only in the *yps1*Δ and *yps7*Δ backgrounds; *yps1*Δ*2*Δ, *yps1*Δ*6*Δ, *yps1*Δ*7*Δ, and *yps6*Δ*7*Δ all showed increased sensitivity to caspofungin and Congo Red compared to the parental strains, whereas *yps2*Δ*7*Δ showed increased sensitivity to calcofluor white only. *yps1*Δ*2*Δ was the most severely affected of the double mutants, with no growth at the lowest concentrations of all four of the drugs. The *yps1*Δ*2*Δ mutant strain also was the only double mutant to show a synthetic effect in response to elevated temperature and caffeine. The genetic interactions between the yapsins suggest that *YPS2* and *YPS6* buffer the cell wall defects that result from the loss of *YPS1* and *YPS7*. This buffering appears to be particularly significant in the interaction of *YPS1* and *YPS2*, suggesting that these two proteases may have partially overlapping functions or substrates.

Yapsin deletion mutants have decreased 1,3- and 1,6-β-glucan and basal activation of the *PKC1-MPK1* pathway. The sensitivity of yapsin mutants to cell wall-disrupting agents as well as the temperature-induced lysis of strains with multiple yapsin mutations suggested that the loss of yapsin function may lead to alteration in the structure or composition of the cell wall. To test this hypothesis, we determined the amounts of 1,3- and 1,6-β-glucan, chitin, and mannan in cell walls isolated from wild type as well as *yps1*Δ2Δ and 5*yps*Δ mutants (Table 5). The cells were grown overnight in the presence of ^{14}C-labeled glucose to establish an equilibrium distribution of label within the four polysaccharide components of the cell wall (40). 5*yps*Δ cell walls had dramatically altered cell wall composition. Both 1,3- and 1,6-β-glucan were significantly reduced in the 5*yps*Δ mutant relative to wild-type cells, with 1,3-β-glucan decreased by 43% and 1,6-β-glucan decreased by 31% (Table 2). *yps1*Δ*2*Δ cells also had decreased 1,3- and 1,6-β-glucan, but consistent with their less severe phenotypic changes, the levels were higher than in the 5*yps*Δ mutant. The decrease in glucan levels in the 5*yps*Δ mutant is similar to those observed in the *fks1*Δ mutant (40), indicating that loss of yapsin function leads to alterations in cell wall composition comparable to those resulting from the loss of a key subunit in the synthase of a cell wall constituent. Reintroduction of *YPS2* to 5*yps*Δ cells led to a partial restoration of 1,3- and 1,6-β-glucan levels as well as a slight reduction in chitin levels. These measurements are consistent with the ability of *YPS2* to rescue the temperature sensitivity of 5*yps*Δ (Fig. 1B).

Although mannan levels were similar to wild type in the 5*yps*Δ mutant, the cell wall chitin content was nearly double that of wild type. Chitin synthesis is increased during periods of cell wall stress and in mutants with alterations in other components of the cell wall (55). Therefore, the increase in chitin levels observed in the 5*yps*Δ mutant is most likely a response to the glucan deficits in the cell wall (59). Cell wall stress-induced increases in chitin are at least partly mediated by a MAP kinase cascade, the cell wall integrity pathway (*PKC1-MPK1* pathway [24, 28, 63]). Consistent with this analysis, the *PKC1-MPK1* pathway is activated during vegetative growth at ambient temperature in the 5*yps*Δ mutant as shown by detection of phosphorylated Mpk1p at 30°C by immunoblotting with phosphospecific antibodies (Fig. 3A). Similar basal activation of Mpk1p was also detected in *yps1*Δ*2*Δ (data not shown). Basal phosphorylation of Mpk1p at ambient temperatures has been reported previously for a variety of cell wall mutants (e.g., *gas1*Δ, *fks1*Δ, and *kre9*Δ [13]). Therefore, the activation of Mpk1p in the yapsin mutants underscores the significance of the cell wall alterations that result from loss of yapsin function.

A second MAP kinase signaling cascade known as the sterile vegetative growth pathway (SVG) (12, 37) has been found to contribute to the maintenance of cell wall integrity in *S. cerevisiae*. The SVG cascade is an amalgamation of components of the pheromone response and high osmolar glycerol MAP kinase pathways. It appears to function in parallel with the *PKC1-MPK1* pathway and up-regulates *FKS2* in response to defects in cell wall protein mannosylation (37). This pathway, however, does not exhibit increased basal activity in the 5*yps*Δ mutant as indicated by the fact that no phosphorylated Kss1p (the MAP kinase of the SVG pathway) was detected in 5*yps*Δ cells during log-phase growth at 30°C (Fig. 3B). Although the stimuli that trigger SVG activation are not well characterized beyond mutants affecting mannoprotein glycosylation (37), this result is consistent with a lack of change in the mannan content of the cell wall in the 5*yps*Δ background.

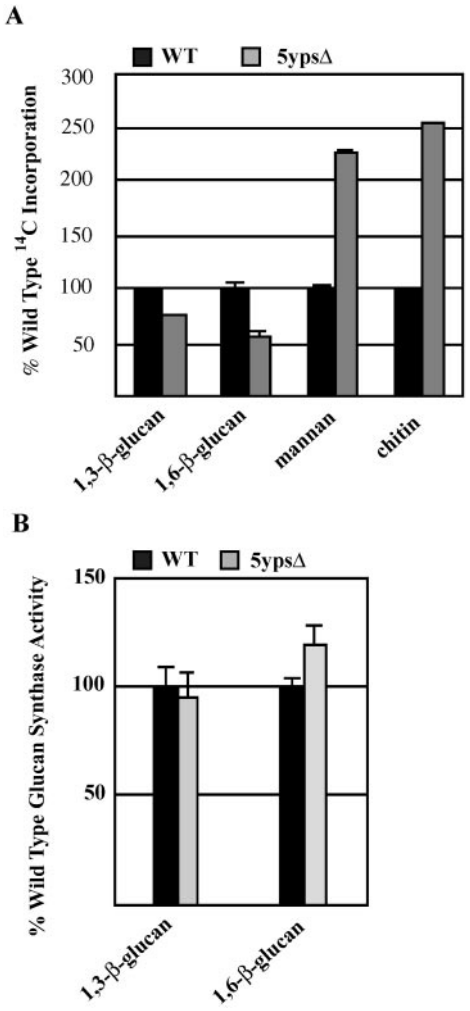

FIG. 4. Incorporation of 1,3- and 1,6-β-glucan is decreased in the 5*yps*Δ mutant, but the specific activity of 1,3- and 1,6-β-glucan synthesis is not. (A) Log-phase cultures of wild-type (WT) and 5*yps*Δ cells were pulse-labeled with [^{14}C]glucose for 5 min. The cell walls were isolated and processed as described in Materials and Methods to determine the amount of label uptake. (B) The specific activity of 1,3- and 1,6-β-glucan synthase was determined as described in Materials and Methods. Extracts were prepared from cells harvested in log phase at 30°C. Data are expressed as percentage of wild-type incorporation/specific activity and are the means of two independent experiments performed in duplicate.

Yapsin mutants have defects in cell wall glucan incorporation but not in glucan synthesis. To determine if the decreased levels of 1,3- and 1,6-β-glucan were due to defects in biosynthesis or incorporation of glucan into the cell wall, we performed pulse-labeling experiments in which cells in early exponential phase were briefly exposed to uniformly labeled glucose. Because actively dividing cells are continuously generating new cell wall, the amount of incorporated label is a direct measure of new glucan synthesized and incorporated into the cell wall. As shown in Fig. 4A, there was decreased uptake of ^{14}C-labeled glucose into both 1,3-β-glucan and 1,6-β-glucan in 5*yps*Δ, but increased ^{14}C-labeled glucose uptake in mannan and chitin relative to wild-type cells at 23°C. As discussed above, the increased mannan and chitin uptake is likely part of a compensatory response to the glucan defects.

In order to determine if yapsin mutations directly affected glucan synthesis, we compared the 1,3-β-glucan and 1,6-β-glucan synthase activities of the 5*yps*Δ mutant and wild-type cells in vitro. As shown in Fig. 4B, the 1,3-β-glucan synthase activity of 5*yps*Δ was essentially identical to that of wild-type cells. Indeed, the in vitro 1,6-β-glucan synthase activity of the 5*yps*Δ mutant strain is slightly increased relative to wild type. These results indicate that the decrease in incorporation of 1,3- and 1,6-β-glucan in the 5*yps*Δ mutants in vivo is not the result of a decrease in glucan synthase activity. A reasonable interpretation of these results is that the yapsins affect glucan homeostasis downstream of polymer synthesis and, therefore, may be involved in facilitating the incorporation of glucan into the cell wall or promoting the retention of previously synthesized glucan within the cell wall.

***YPS1* expression is induced by cell wall stress and remodeling.** As an initial investigation into the basis for the differential roles of the yapsins in cell wall integrity, we focused on Yps1p and hypothesized that it may be part of the transcriptional response to cell wall stress. Ash and coworkers had previously observed that during growth at 37°C Yps1p levels are increased 12-fold relative to ambient temperature (2), suggesting that *YPS1* expression may be induced during cell wall stress. We also hypothesized that *YPS1* might be expressed during periods of increased glucan synthesis, based on the yapsins' apparent role in glucan homeostasis. In order to test these assertions, we constructed a reporter plasmid with *lacZ* fused to the intergenic region 5′ to *YPS1* and examined the expression of *YPS1* under conditions that either cause cell wall stress, require cell wall remodeling, or increase expression of the inducible subunit of 1,3-β-glucan synthase, *FKS2* (67). Consistent with the findings of Ash et al. (2) and Sievi et al. (58), expression of *YPS1* was extremely low at 25°C but increased fourfold upon shift to 37°C in YPD (Table 6). Treatment of wild-type cells with zymolyase, a 1,3-β-glucanase-containing enzyme preparation, increased *YPS1* expression sevenfold. The cell wall-perturbing dyes Congo Red and calcofluor white also strongly increased *YPS1* expression, confirming that *YPS1* is induced by cell wall-damaging conditions.

In order to test the hypothesis that the yapsins may be important during times of increased glucan synthesis, we asked if the expression of *YPS1* was induced by conditions that also increase the expression of *FKS2* (67). *FKS2* expression is increased by extracellular calcium, growth on poor carbon sources, and exposure to α-factor, the mating pheromone for *S. cerevisiae*. As shown in Table 6, all of these conditions also increase *YPS1* expression. Finally, *YPS1* expression was dramatically induced at early stationary phase, a period of extensive cell wall remodeling that is also characterized by high levels of *FKS2* expression (67). These results indicate that *YPS1* expression is induced during periods of cell wall stress and/or remodeling in a manner that appears to closely parallel the expression of the inducible subunit of glucan synthase, *FKS2*.

In order to assess further the functional significance of increased *YPS1* expression, we examined the synthetic genetic interactions between *YPS1* and *CBK1*. Cbk1p is a protein kinase involved in the regulation of polarized cell growth and

TABLE 6. Expression of β-galactosidase from *YPS1-lacZ* reporter plasmid

Strain[a]	Growth condition[b]	Miller units[c]	Fold change[d]
W303			
CRY1	25°C	0.9 (0.3)	1.0
CRY1	37°C (12 h)	3.8 (1.3)	4.2
CRY1	α-factor[e]	3.8 (0.7)	4.2
CRY1	Zymolyase[f]	6.7 (0.7)	7.4
CRY1	CR[g]	10.8 (1.7)	12
CRY1	CFW_{20}[h]	12.3 (3.2)	14
CRY1	YPGal[i]	4.1 (0.3)	4.6
CRY1	$CaCl_2$[j]	16.2 (1.2)	18
CRY1	SP[k]	53.0 (11)	59
*cbk1*Δ	25°C	5.6 (0.4)	6.2
S288C			
BY4741	25°C	1.3 (0.4)	1.0
BY4741	CFW_{10}[l]	5.3 (0.6)	4
*mpk1*Δ	25°C	2.8 (0.3)	2.2
*mpk1*Δ	CFW_{10}	2.2 (0.4)	1.7
*rml1*Δ	25°C	1.3 (0.4)	1.0
*rml1*Δ	CFW_{10}	6.0 (0.5)	4.6
*crz1*Δ	25°C	0.9 (0.3)	0.7
*crz1*Δ	$CaCl_2$	0.4 (0.1)	0.3

[a] Background of parent strain (see Table 1 for full details).
[b] All incubations were at 25°C in YPD for 5 h containing the indicated supplement unless indicated otherwise. Cells were harvested in mid-logarithmic phase (OD_{600} of <1.0) unless otherwise indicated.
[c] Miller unit = nmol of *o*-nitrophenol/mg/min at 37°C.
[d] Fold change relative to wild type at 25°C in YPD.
[e] 0.8 μg/ml α-factor (>90% cells in G_0 or shmooing by light microscopy).
[f] 20 U/ml 100T Zymolyase.
[g] 50 μg/ml Congo Red (CR).
[h] 20 μg/ml calcofluor white (CFW).
[i] YPGal refers to yeast peptone (YP) containing 2% galactose.
[j] 30 mM $CaCl_2$.
[k] Early stationary phase; incubation in SDC-URA for 3 days at 25°C.
[l] 10 μg/ml CFW.

morphogenesis, possibly through activation of the transcription factor Ace2p (65). Genome-wide transcriptional profiling experiments have shown that deletion of *CBK1* results in the up-regulation of a number of genes involved in cell wall processes including *YPS1* (4). Using the pr*YPS1-lacZ* reporter, we determined the level of *YPS1* expression in the *cbk1*Δ mutant and found it to be ~6-fold increased relative to wild type at ambient temperature (Table 6). Next, we compared the growth of *yps1*Δ*cbk1*Δ to that of both *yps1*Δ and *cbk1*Δ in the presence of calcofluor white and Congo Red. *cbk1*Δ is hypersensitive to both of these agents at high concentrations, suggesting that the cell wall is affected by this mutation (data not shown). At low concentrations of these drugs, *yps1*Δ*cbk1*Δ showed increased sensitivity to calcofluor white (Fig. 5) and Congo Red (data not shown) relative to the single mutants (Fig. 5), suggesting that *YPS1* plays a role in compensating for the cell wall defects caused by the *cbk1*Δ mutation. Taken together, these data indicate that *YPS1* is part of the transcriptional response to cell wall stress and that Yps1p plays a significant role in preserving cell wall integrity during stress.

***YPS1* expression is regulated by both the cell wall integrity pathway and calcineurin.** Because *YPS1* is induced during cell wall stress, we asked if *YPS1* is part of the transcriptional output of the *PKC1-MPK1* pathway, the best-characterized signaling cascade involved in controlling the cellular response to cell wall stress (24). Toward that end, we introduced the

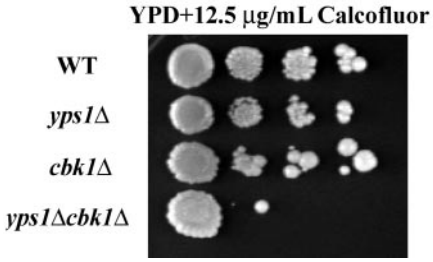

FIG. 5. Synthetic genetic interaction between *yps1*Δ and *cbk1*Δ. Serial 10-fold dilutions of a 1-OD_{600} suspension of the indicated strains were pronged onto plates containing YPD plus 12.5 μg/ml calcofluor white, grown for 4 days at 30°C, and photographed. WT, wild type.

pr*YPS1-lacZ* reporter construct into strains with null mutations in *MPK1*, the terminal MAPK of the cell wall integrity pathway, and *RLM1*, a transcription factor in the same pathway (29). Treatment of cells with calcofluor white induced expression of *YPS1* in both wild-type and *rlm1*Δ strains but not in the *mpk1*Δ mutant (Table 6). Therefore, induction of *YPS1* expression in response to cell wall damage requires the *PKC1-MPK1* pathway but is independent of *RLM1*. This observation supports a growing body of evidence for the existence of *PKC1-MPK1*-activated transcription factors in addition to Rlm1p and, once again, recalls the Mpk1-dependent, Rlm1-independent expression pattern of *FKS2* (24, 29). Interestingly, baseline expression of *YPS1* was slightly increased in the *mpk1*Δ mutant relative to wild type, suggesting that other signaling pathways that respond to cell wall stress may also increase *YPS1* expression. Because *FKS2* is coregulated by the *PKC1-MPK1* pathway and by calcineurin-dependent signaling (67), we also determined if *YPS1* expression was regulated by calcineurin. Indeed, induction of expression of *YPS1* by extracellular calcium was abolished by deletion of *CRZ1*, which encodes the calcineurin-activated transcription factor (60). Taken together, these results indicate that Yps1p is a component of the transcriptional response to cell wall stress and that, like the stress-induced glucan synthase subunit *FKS2*, *YPS1* is coregulated by *PKC1-MPK1* and calcineurin.

Overexpression of *YPS1* suppresses caspofungin hypersensitivity. In order to gain further insight into the function of *YPS1*, we determined the effects of overexpression of *YPS1*. To this end, we expressed *YPS1* from the strong *TDH* promoter on a 2μ-plasmid (pG*YPS1*) under a variety of conditions. Wild-type cells expressing *YPS1* at high levels showed no toxic effects under a variety of conditions including rich and synthetic growth media, elevated temperature, or presence of calcofluor white or caffeine. In fact, wild-type cells containing pG*YPS1* were more resistant to high concentrations of both caspofungin (Fig. 6A) and Congo Red (data not shown) than were those harboring an empty vector. This suggested that overexpression of *YPS1* might promote the stability of the cell wall under conditions that disrupt normal glucan homeostasis. To test this possibility further, pG*YPS1* was transformed into a set of mutants that display caspofungin hypersensitivity (38), including strains with mutations of genes involved in 1,3-β-glucan synthesis (*fks1*Δ and *smi1*Δ), chitin synthesis (*chs3*Δ), and mannoprotein glycosylation (*mnn10*Δ). In all cases, pG*YPS1* suppressed the caspofungin hypersensitivity of those mutants (Fig.

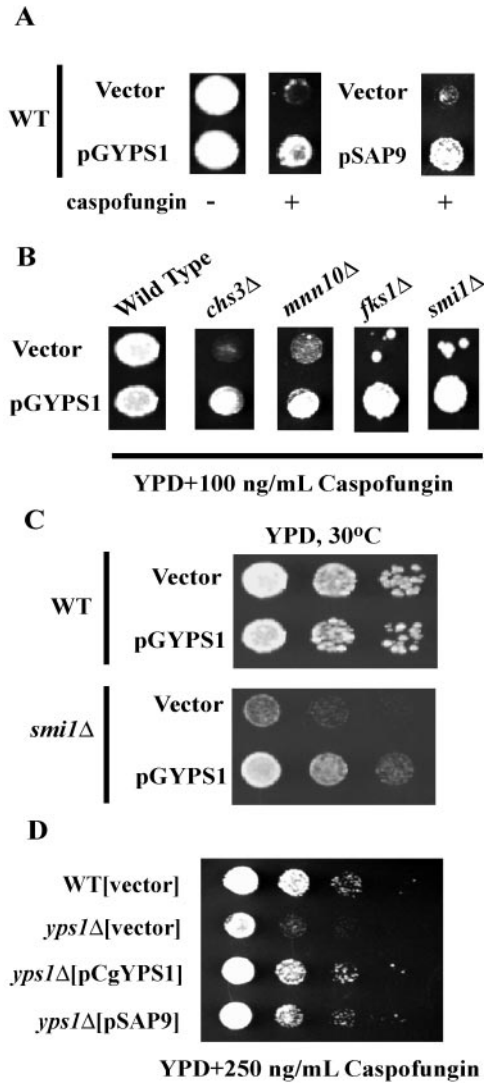

FIG. 6. Overexpression of *YPS1* induces caspofungin tolerance in wild-type cells and suppresses phenotypes of cell wall mutants. (A) A suspension of wild-type (WT) cells harboring either a 2μ-plasmid or *YPS1*/*SAP9* in the same plasmid under a constitutive promoter was spotted on YPD supplemented with 500 ng/ml of caspofungin. (B) Serial 10-fold dilutions of either wild type or the indicated cell wall mutants containing pG5 or pGYPS1 were pronged on YPD and incubated at 30°C for 2 days. (C) Serial 10-fold dilutions of wild-type or *smi1*Δ strains containing pG5 or pG*YPS1* were spotted on YPD and incubated at 30°C for 2 days. Yapsin homologues from *Candida albicans* (*SAP9*) and *Candida glabrata* (*CgYPS1*) complement the caspofungin sensitivity of *yps1*Δ. (D) Serial 10-fold dilutions of wild-type and *yps1*Δ cells harboring pRS426-GPD, pRS426-GPD-*SAP9*, or pRS416-*CgYPS1* were pronged on YPD plates supplemented with 100 ng/ml of caspofungin and incubated at 30°C for 3 days.

6B), indicating that increased expression of *YPS1* leads to a decreased dependence on 1,3-β-glucan synthesis for maintenance of cell wall integrity.

The ability of high-level expression of *YPS1* to compensate for decreased 1,3-β-glucan synthesis is further supported by the effect of *YPS1* overexpression on the growth of *smi1*Δ. Smi1p, also known as Knr4p, is a nuclear protein that appears to be an important regulator of 1,3-β-glucan synthesis, although its exact mechanism of action is unclear (25, 41). The *smi1*Δ mutant displays a slow-growth phenotype at 30°C and has significantly reduced amounts of 1,3-β-glucan in the cell wall as well as a corresponding decrease in 1,3-β-glucan synthase specific activity (25). Overexpression of *YPS1* in the *smi1*Δ background not only suppresses caspofungin sensitivity but also partially suppresses the slow-growth defect (Fig. 6B). This indicates that the positive effect of *YPS1* overexpression on glucan-deficient/dependent mutants is not due to an idiosyncratic interaction with caspofungin and further supports the notion that the increased expression of *YPS1* during cell wall stress plays an important role in maintaining cell wall glucan homeostasis.

***YPS1* homologues in *Candida albicans* and *Candida glabrata* complement *yps1*Δ mutants.** Close homologues of *YPS1* have been identified in two species of *Candida*, *C. albicans* and *C. glabrata*. A large family of secreted aspartyl proteases (*SAP* genes) is present in *C. albicans* and has been the focus of intensive study, particularly with respect to virulence and colonization (46). Monod et al. identified a putative GPI-linked *SAP*, *SAP9*, and showed that it was more closely related to *YPS1* and *YPS2* than to the other eight *SAP* genes (*SAP1* to *-8*) (43). In addition, the genome of *C. glabrata* was sequenced recently and a number of yapsin homologues are apparent, including an open reading frame (CAGLOM0404191g) that closely resembles *YPS1* ($P = 2e{-}82$ [16]). Since little is known about the function of these yapsin homologues, we cloned *SAP9* and CAGLOM0404191g, hereafter referred to as *CgYPS1*, into expression vectors (pRS416 and pRS416-GPD) suitable for *S. cerevisiae* and asked if these constructs would complement the phenotypes of the *yps1*Δ mutants. Although *SAP9* expressed from its endogenous promoter would not complement *yps1*Δ phenotypes, expression of *SAP9* under the control of a constitutive promoter, *GPD*, complemented the caspofungin (Fig. 6C) and Congo Red sensitivity but not the caffeine sensitivity of the *yps1*Δ mutant (data not shown). Like *YPS1*, overexpression of *SAP9* in wild-type cells also increased tolerance to caspofungin (Fig. 6A). In the case of the *C. glabrata* *YPS1* homologue, expression of *CgYPS1* under its own promoter was sufficient to complement all three of the cell wall phenotypes of *yps1*Δ. Both of these observations suggest that the yapsin homologues in *Candida* spp. may also be involved in cell wall integrity and that yapsin-mediated processes could be a general requirement for proper cell wall homeostasis in yeast.

DISCUSSION

Prior to this work, the yapsins represented a major family of proteases in *S. cerevisiae* for which no clear physiological function was known. In this report, we have demonstrated that the yapsins are required for cell wall integrity and appear to be important for maintaining cell wall glucan homeostasis. The yapsins are a family of relatively closely related proteases and appear to have at least some overlapping functions based on the cumulative effects of multiple yapsin mutations on cell wall-related phenotypes. Our data, however, also indicate that the yapsins are not simply a family of redundant proteases and, instead, that the yapsins have differentiable roles in cell wall integrity. Of the single yapsin mutants, *yps1*Δ and *yps7*Δ are the

only mutants that display significant phenotypes, suggesting that they play particularly important roles in cell wall processes. Further supporting this notion is the fact that synthetic phenotypes were observed only in double mutants within either the *yps1*Δ or *yps7*Δ background. Using a reporter plasmid to examine the expression of *YPS1* under a variety conditions, we have shown that *YPS1* is part of the transcriptional response to cell wall stress mediated by the cell wall integrity pathway and, thereby, have provided a reasonable explanation for the prominent role of *YPS1* in cell wall integrity.

Multiple genome-wide studies of the transcriptional response to cell wall stress suggest that the expression of *YPS7* is relatively unchanged and, actually, may be repressed during such conditions (7, 20, 21, 27). Therefore, it appears that expression during cell wall stress may not be the only factor that determines which yapsins are important for cell wall integrity. An intriguing alternative explanation for the prominent role of *YPS7* is related to its possible localization. Because the yapsins are GPI-linked proteins, they have two possible localizations: the plasma membrane and the cell wall (30). *YPS1*, *-2*, *-3*, and *-6* all have C-terminal sequence motifs consistent with plasma membrane localization (18, 23), and in the cases of Yps1p and Yps2p, such localization has been confirmed experimentally (2, 32, 58). On the other hand, the C-terminal motif in *YPS7* is suggestive of a cell wall-localized GPI protein (23). Yps7p may, therefore, be exposed to a unique pool of substrates of crucial importance to cell wall integrity, and future studies will examine the possible role that localization plays in determining yapsin function.

The only yapsin mutant that did not have either a cell wall-related phenotype or a synthetic interaction with other yapsin mutants was *yps3*Δ. The apparently limited role that *YPS3* plays in cell wall processes was somewhat surprising because *YPS3* has been shown to be up-regulated by temperature (21), cell wall-related gene mutation (61), and antifungal drugs (1). *YPS3* does have some function in cell wall integrity because the *yps1*Δ*2*Δ*3*Δ mutant has a more severe growth defect at 37°C than the *yps1*Δ*2*Δ mutant, but its role is clearly secondary to the other yapsins. One explanation for the secondary role for *YPS3* may be that it has other functions that are indirectly involved in maintaining cell wall integrity, possibly as part of a more generalized stress response. It is also intriguing that *YPS1* and *YPS3* are contiguous genes in the genome, suggesting the possibility that *YPS1* and *YPS3* represent a gene duplication and that *YPS3* is the less functional of the pair with respect to cell wall processes.

Among the double yapsin mutants, the most severely affected is the *yps1*Δ*2*Δ mutant. *yps1*Δ*2*Δ is the only double mutant that displays temperature sensitivity and, like the quintuple yapsin mutant, 5*yps*Δ, has an altered cell wall composition. The significant synthetic genetic interaction of *YPS1* and *YPS2* suggests that some overlapping function between the two proteases may exist. Previous work in our lab showed that whole cell-associated proteolytic activity toward fluorogenic model yapsin substrates is decreased by 80% in the *yps2*Δ mutant, while it is little changed in the *yps1*Δ mutant (34). We have also found that HA-tagged Yps2p is readily detectable in extracts of log-phase cells at 30°C by Western blotting (D. J. Krysan and R. S. Fuller, unpublished results), while Sievi et al. found that Yps1p is barely detectable by immunoblotting unless the cells are incubated at 37°C (58). Taken together, these observations suggest that Yps2p is constitutively expressed, and active, in vegetative cells under ambient conditions and that Yps1p is expressed during times of cell wall stress. Yps1p appears to be sufficient to compensate for the loss of Yps2p with respect to maintaining cell wall integrity, but the fact that *yps1*Δ shows cell wall compromise suggests that Yps1p has functions in addition to those that overlap with Yps2p. When both Yps1p and Yps2p are lost, the cell wall structure is significantly altered, apparently because of the disruption of a set of processes crucial for the maintenance of glucan homeostasis.

In general terms, glucan homeostasis can be divided into three sequential stages: (i) synthesis, (ii) cell wall incorporation, and (iii) remodeling or turnover (15, 54, 59). The important role of the yapsins in glucan homeostasis is clearly demonstrated by the significant reduction in 1,3- and 1,6-β-glucan in the *yps1*Δ*2*Δ and 5*yps*Δ mutants and the decreased rate of new 1,3- and 1,6-β-glucan incorporation in the 5*yps*Δ mutant relative to wild type. The yapsins appear to affect stages of glucan homeostasis downstream of initial polymer synthesis because the in vitro specific activity of the 1,3- and 1,6-β-glucan synthases is not decreased in the 5*yps*Δ mutant. Therefore, the yapsins seem to function in glucan homeostasis either by facilitating the incorporation of newly synthesized glucan into the cell wall or by promoting the retention of glucan within the cell wall. Distinguishing between these two possibilities will require the identification and characterization of bona fide yapsin substrates. At this time, few details are known regarding the proteins involved in transport of newly synthesized glucan across the plasma membrane or the incorporation of this glucan into the cell wall (38, 54). Therefore, it is difficult to speculate as to the identity of potential yapsin substrates involved in these processes.

In terms of the other possible functions for the yapsins, retention of glucan within the cell wall, one possible set of substrates does seem evident: cell wall glucanases. A number of actual and putative glucanases and transglucosidases have been identified in *S. cerevisiae* (3, 6, 10, 44, 57). Again, few details regarding their specific functions are known, but it is presumed that the glucanases and transglucosidase are involved in remodeling of the cell wall (59). The cell wall is a dynamic structure, and the maintenance of cell wall integrity is the result of a complex network of regulatory processes that modulate a balance between synthetic and degradative processes (10). The yapsins could regulate the activity of cell wall glucanases or other hydrolytic enzymes through programmed, or selective, degradation. In this model, during periods of increased glucan synthesis the yapsins may act to decrease the turnover of glucan by degrading the glucanases and, thereby, promoting the retention of glucan within the cell wall. In principle, either 1,3- or 1,6-β-glucan could be affected by loss of the yapsins in such a model. At this time, it is not possible to deduce whether the yapsins primarily affect 1,3- or 1,6-β-glucan, or both, because deletion of genes involved in cell wall integrity can lead to any of the four possible combinations of increased and/or decreased 1,3- and 1,6-β-glucan (14, 51).

Our initial investigations of the factors that distinguish the roles of specific yapsins in cell wall integrity have focused on *YPS1*, and we have shown that *YPS1* is part of the transcrip-

tional response to cell wall stress. We have also shown that *YPS1* expression parallels that of *FKS2*, the inducible subunit for 1,3-β-glucan synthesis. These observations suggest a model in which *YPS1* promotes either glucan incorporation or retention during periods of increased glucan synthesis in response to cell wall stress and remodeling. Such a model is also consistent with the observation that overexpression of *YPS1* suppresses both inhibitor- and mutation-induced defects in 1,3-β-glucan synthesis. In other words, increased *YPS1* expression appears to decrease the amount of 1,3-β-glucan synthesis required to maintain cell wall integrity.

The role of yapsin-like proteases in cell wall integrity does not appear to be limited to *S. cerevisiae*, because we have found that homologues of *YPS1* in two pathogenic *Candida* spp., *C. albicans* and *C. glabrata*, can complement the cell wall defects of the *yps1*Δ mutant. Unlike Sap1- to -8p in *C. albicans*, Sap9p is apparently not secreted, and like *YPS1*, *SAP9* expression is increased during stationary phase and in response to cell wall perturbation (11, 43), adding support to the notion that *YPS1* and *SAP9* may have similar functions. Additionally, the sequence specificity of Yps1p and Sap9p appears to be reasonably similar because a peptide isostere-based inhibitor of Yps1p also has activity against Sap9p (9). Together, these considerations suggest that Sap9p may carry out similar functions in *C. albicans*. A key difference between the abilities of *SAP9* and *CgYPS1* (the *C. glabrata YPS1* homologue) to complement the *yps1*Δ mutant is that *SAP9* did so only when expressed from a constitutive, heterologous promoter while the wild-type promoter of *CgYPS1* allowed complementation of the *yps1*Δ mutant. One possible explanation for this observation is that *C. glabrata* is more closely related to *S. cerevisiae* than to other *Candida* spp. and, therefore, either the protease specificity or the regulation of *CgYPS1* expression matches *YPS1* more closely than does that of *SAP9*.

Further supporting a functional parallel between Sap9p and Yps1p is the fact that overexpression of either gene leads to resistance to the clinically important antifungal caspofungin. Prior to this work, *SBE2*, a Golgi protein involved in the transport of cell wall components, was the only other reported multicopy suppressor of caspofungin toxicity in *S. cerevisiae* (50). To our knowledge, this is the first report of a *C. albicans* gene that is a multicopy suppressor of caspofungin toxicity. Therefore, elucidation of the mechanism of Yps1p/Sap9p-induced caspofungin tolerance not only will have relevance to fundamental processes in cell wall biology but may also have importance for potential resistance mechanisms related to caspofungin, an important component of the antifungal armamentarium.

ACKNOWLEDGMENTS

We thank David Levin (Johns Hopkins), Amy Tong and Charlie Boone (Toronto), Frans Klis (Amsterdam), Dennis Thiele (Duke), K.-L. Guan (UM), Michael Monod (Lausanne), and David Engelke (UM) for generously providing plasmids, strains, or reagents. Paula Magnelli (Boston U.) performed preliminary cell wall analyses.

This work was supported by a PIDS Fellowship funded by Roche (D.J.K.) and the following National Institutes of Health grants: T32 HD007513 (D.J.K.), R01 GM59773 (C.A.), and R01 GM39697 (R.S.F.). L.K. thanks R.S.F. for hosting his sabbatical, which was supported by the Michigan Agricultural Experiment Station and NIH GM43585.

REFERENCES

1. **Agarwal, A. K., P. D. Rogers, S. R. Baerson, M. R. Jacob, K. S. Barker, J. D. Cleary, L. A. Walker, D. G. Nagle, and A. M. Clark.** 2003. Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. J. Biol. Chem. **278:** 34998–35015.
2. **Ash, J., M. Dominguez, J. J. M. Bergeron, D. Y. Thomas, and Y. Boubonnais.** 1995. The yeast proprotein convertase encoded by *YAP3* is a glycophosphatidylinositol-anchored protein that localizes to the plasma membrane. J. Biol. Chem. **270:**20847–20854.
3. **Basco, R. D., G. Gimenez-Gallego, and G. Larriba.** 1990. Processing of yeast exoglucanase (beta-glucosidase) in a *KEX2*-dependent manner. FEBS Lett. **268:**99–102.
4. **Bidlingmaier, S., E. L. Weiss, C. Seidel, D. G. Drubin, and M. Snyder.** 2001. The Cbk1p pathway is important for polarized cell growth and cell separation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **21:**2449–2462.
5. **Burke, D. J., D. Dawson, and T. Stearns.** 2000. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Woodbury, N.Y.
6. **Cappellaro, C., V. Mrsa, and W. Tanner.** 1998. New potential cell wall glucanases of *Saccharomyces cerevisiae* and their involvement in mating. J. Bacteriol. **180:**5030–5037.
7. **Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Lander, and R. A. Young.** 2001. Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell **12:**323–337.
8. **Cawley, N. X., H.-C. Chen, M. C. Beinfeld, and Y. P. Loh.** 1996. Specificity and kinetic studies on the cleavage of various prohormone mono- and paired-basic residue sites by yeast aspartic protease 3. J. Biol. Chem. **271:** 4168–4176.
9. **Cawley, N. X., M. Chino, A. Maldonado, Y. M. Rodriquez, Y. P. Loh, and J. A. Ellman.** 2003. Synthesis and characterization of the first potent inhibitor of yapsin 1. J. Biol. Chem. **278:**5523–5530.
10. **Cid, V. J., A. Duran, F. del Ray, M. P. Snyder, C. Nombela, and M. Sanchez.** 1995. Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **59:**345–386.
11. **Copping, V. M. S., C. J. Barelle, B. Hube, N. A. R. Gow, A. J. P. Brown, and F. C. Odds.** 2005. Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. J. Antimicrob. Chemother. **55:**645–654.
12. **Cullen, P. J., J. Schultz, J. Horecka, B. J. Stevenson, Y. Jigami, and G. F. Sprague, Jr.** 2000. Defects in protein glycosylation cause *SHO1*-dependent activation of a *STE12* signaling pathway in yeast. Genetics **155:**1005–1018.
13. **de Nobel, H., C. Ruiz, H. Martin, W. Morris, S. Brul, M. Molina, and F. M. Klis.** 2000. Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in *FKS2-lacZ* expression, glucanase resistance and thermotolerance. Microbiology **146:** 2121–2132.
14. **Dijkgraaf, G. J. P., M. Abe, Y. Ohya, and H. Bussey.** 2002. Mutations in Fks1p affect the cell wall content of β-1,3- and β-1,6-glucan in *Saccharomyces cerevisiae*. Yeast **51:**559–564.
15. **Douglas, C. M.** 2001. Fungal β(1,3)-D-glucan synthesis. Med. Mycol. **39**(Suppl. 1)**:**55–66.
16. **Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Souciet.** 2004. Genome evolution in yeasts. Nature **430:**35–44.
17. **Egel-Mitani, M., H. P. Flygenring, and M. T. Hansen.** 1990. A novel aspartyl protease allowing *KEX2*-independent MF alpha propheromone processing in yeast. Yeast **6:**127–137.
18. **Frieman, M. B., and B. P. Cormack.** 2003. The ω-site sequence of glycosylphosphatidylinositol-anchored proteins in *Saccharomyces cerevisiae* can determine the distribution between the membrane and the cell wall. Mol. Microbiol. **50:**883–896.
19. **Frost, D. J., K. Brandt, J. Capobianco, and R. Goldman.** 1994. Characterization of (1,3)-β-glucan synthase in *Candida albicans*: microsomal assay from the yeast or mycelial morphological forms and a permeabilized whole-cell assay. Microbiology **140:**2239–2246.
20. **Garcia, R., C. Bermejo, C. Grau, R. Perez, J. M. Rodriguez-Pena, J. Francois, C. Nombela, and J. Arroyo.** 2004. The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. J. Biol. Chem. **279:**15183–15195.
21. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Havel, M. B. Eisen, D.**

Botstein, G. Storz, and P. O. Brown. 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell **11:** 4241–4257.
22. **Gustin, M. C., J. Albertyn, M. Alexander, and K. Davenport.** 1998. MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **62:**1264–1300.
23. **Hamada, K., H. Terashima, M. Arisawa, N. Yabuki, and K. Kitada.** 1999. Amino acid residues in the ω-minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. J. Bacteriol. **181:**3886–3889.
24. **Heinisch, J. J., A. Lorberg, H.-P. Schmitz, and J. J. Jacoby.** 1999. The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. Mol. Microbiol. **32:** 671–680.
25. **Hong, Z., P. Mann, N. H. Brown, L. E. Tran, K. J. Shaw, R. S. Hare, and B. DiDomenico.** 1994. Cloning and characterization of *KNR4*, a yeast gene involved in (1,3)-β-glucan synthesis. Mol. Cell. Biol. **14:**1017–1025.
26. **Hughes, T. R., M. J. Marton, A. R. Jones, C. J. Roberts, R. Stoughton, C. D. Armour, H. A. Bennett, E. Coffey, H. Dai, Y. D. He, M. J. Kidd, A. M. King, M. R. Meyer, D. Slade, P. Y. Lum, S. B. Stepaniants, D. D. Shoemaker, D. Gachotte, K. Chakraburtty, J. Simon, M. Bard, and S. H. Friend.** 2000. Functional discovery via a compendium of expression profiles. Cell **7:**109–126.
27. **Jung, U. S., and D. E. Levin.** 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signaling pathway. Mol. Microbiol. **34:**1049–1057.
28. **Jung, U. S., A. K. Sobering, M. J. Romeo, and D. E. Levin.** 2002. Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. Mol. Microbiol. **46:**781–789.
29. **Kamada, Y., U. S. Jung, J. Piotrowski, and D. E. Levin.** 1995. The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. Genes Dev. **9:**1559–1571.
30. **Kapteyn, J. C., H. Van Den Ende, and F. M. Klis.** 1999. The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim. Biophys. Acta **1426:**373–383.
31. **Klis, F. M., P. Mol, K. Hellingwerf, and S. Brul.** 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **26:**239–256.
32. **Komano, H., and R. S. Fuller.** 1995. Shared functions *in vivo* of a glycosylphosphatidylinositol-linked aspartyl protease, Mkc7, and the proprotein processing protease Kex2 in yeast. Proc. Natl. Acad. Sci. USA **92:**10752–10756.
33. **Komano, H., N. C. Rockwell, G. T. Wang, G. A. Krafft, and R. S. Fuller.** 1999. Purification and characterization of the yeast glycosylphosphatidylinositol-anchored, mono-basic specific aspartyl protease yapsin 2 (Mkc7). J. Biol. Chem. **274:**24431–24437.
34. **Komano, H., M. Seeger, S. Gandy, G. T. Wang, G. A. Krafft, and R. S. Fuller.** 1998. Involvement of cell surface glycosyl-phosphatidylinositol-linked aspartyl proteases in α-secretase-type cleavage and ectodomain solubilization of human Alzheimer β-amyloid precursor protein in yeast. J. Biol. Chem. **273:**31648–31651.
35. **Kopecka, M., and M. Gabriel.** 1992. The influence of congo red on the cell wall and (1–3)-beta-D-glucan microfibril biogenesis in *Saccharomyces cerevisiae*. Arch. Microbiol. **158:**115–126.
36. **Kunihira, S., Y. Kananishi, M. Sano, K. Naito, Y. Matsuura, Y. Taleno, T. Gojohori, Y. Yamagata, K. Abe, and M. Machida.** 2002. A polymerase chain reaction-based method for cloning novel members of a gene family using a combination of degenerate and inhibitory primers. Gene **289:**177–184.
37. **Lee, B. N., and E. A. Elion.** 1999. The MAPKKK Ste11 regulates vegetative growth through a kinase cascade of shared signaling components. Proc. Natl. Acad. Sci. USA **96:**12679–12684.
38. **Lesage, G., A.-M. Sdicu, P. Menard, J. Shapiro, S. Hussein, and H. Bussey.** 2004. Analysis of β-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. Genetics **167:**35–49.
39. **Lum, P. Y., C. D. Armour, S. B. Stepaniants, G. Cavet, M. K. Wolf, J. S. Butler, J. C. Hinshaw, P. Garnier, G. D. Prestwich, A. Leonardson, P. Garrett-Engele, C. M. Rush, M. Bard, G. Schimmack, J. W. Phillips, C. J. Roberts, and D. D. Shoemaker.** 2004. Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. Cell **116:**121–137.
40. **Magnelli, P., J. F. Cipollo, and C. Abeijon.** 2002. A refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and beta-1,6-glucan fine structure. Anal. Biochem. **301:**136–150.
41. **Martin-Yken, H., A. Dagkessamanskaia, F. Basmaji, A. Lagorce, and J. Francois.** 2003. The interaction of Slt2 MAP kinase with Knr4 is necessary for signalling through the cell wall integrity pathway in *Saccharomyces cerevisiae*. Mol. Microbiol. **49:**23–35.
42. **Molina, M., H. Martin, M. Sanchez, and C. Nombela.** 1998. MAP kinase-mediated signal transduction pathways. Methods Microbiol. **26:**375–394.
43. **Monod, M., B. Hube, D. Hess, and D. Sanglard.** 1998. Differential regulation of *SAP8* and *SAP9* which encode two new members of the secreted aspartyl protease family in *Candida albicans*. Microbiology **244:**2731–2737.
44. **Mrsa, V., and W. Tanner.** 1999. Role of NaOH-extractable cell wall proteins Ccw5p, Ccw6p, Ccw7p and Ccw8p (members of the Pir protein family) in stability of the *Saccharomyces cerevisiae* cell wall. Yeast **15:**813–820.
45. **Myers, A. M., A. Tzagoloff, D. M. Kinneny, and C. J. Lusty.** 1986. Yeast shuttle vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene **45:**299–310.
46. **Naglik, J. R., S. J. Challacombe, and B. Hube.** 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. **67:**400–428.
47. **Olsen, V., N. X. Cawley, J. Brandt, M. Egel-Mitani, and Y. P. Loh.** 1999. Identification and characterization of *Saccharomyces cerevisiae* yapsin 3, a new member of the yapsin family of aspartic proteases encoded by the *YPS3* gene. Biochem. J. **339:**407–411.
48. **Olsen, V., K. Guruprasad, N. X. Cawley, H.-C. Chen, T. L. Blundell, and Y. P. Loh.** 1998. Cleavage efficiency of the novel aspartic protease yapsin 1 (Yap3p) enhanced for substrates with arginine residues flanking the P1 site: correlation with electronegative active-site pockets predicted by molecular modeling. Biochemistry **37:**2768–2777.
49. **Orlean, P.** 1997. Biogenesis of yeast wall and surface components, p. 229–363. *In* J. R. Pringle, J. R. Broach, and E. W. Jones (ed.), The molecular and cellular biology of the yeast *Saccharomyces*: cell cycle and cell biology. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
50. **Osherov, N., G. S. May, N. D. Albert, and D. P. Kontoyiannis.** 2002. Overexpression of Sbe2p, a Golgi protein, results in resistance to caspofungin in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **46:**2462–2469.
51. **Page, N., M. Gerard-Vincent, P. Menard, M. Beaulieu, M. Azuma, G. J. P. Dijkgraaf, H. Li, J. Marcoux, T. Nguyen, T. Dowse, A.-M. Sdicu, and H. Bussey.** 2003. A *Saccharomyces cerevisiae* genome-wide killer toxin screen for altered sensitivity to K1 killer toxin. Genetics **163:**875–894.
52. **Ram, A. F. J., A. Wolters, R. Ten Hoopen, and F. M. Klis.** 1994. A new approach for isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. Yeast **10:**1019–1030.
53. **Rockwell, N. C., D. J. Krysan, T. Komiyama, and R. S. Fuller.** 2002. Precursor processing by Kex2/furin proteases. Chem. Rev. **102:**4525–4548.
54. **Roh, D. H., B. Bowers, H. Riezman, and E. Cabib.** 2002. Rho1p mutations specific for regulation of beta(1→3)glucan synthesis and the order of assembly of the yeast cell wall. Mol. Microbiol. **44:**1167–1183.
55. **Roncero, C.** 2002. The genetic complexity of chitin synthesis in fungi. Curr. Genet. **41:**367–378.
56. **Rupp, S.** 2002. *lacZ* assays in yeast. Methods Enzymol. **350:**112–140.
57. **Sestak, S., I. Hagen, W. Tanner, and S. Strahl.** 2004. Scw10p, a cell-wall glucanase/transglucosidase important for cell-wall stability in *Saccharomyces cerevisiae*. Microbiology **150:**3197–3208.
58. **Sievi, E., T. Suntio, and M. Makarow.** 2001. Proteolytic function of GPI-anchored plasma membrane protease Yps1p in the yeast vacuole and Golgi. Traffic **2:**896–907.
59. **Smits, G. J., H. van den Ende, and F. M. Klis.** 2001. Differential regulation of cell wall biogenesis during growth and development in yeast. Microbiology **147:**781–794.
60. **Stathopoulous, A. M., and M. S. Cyert.** 1997. Calcineurin acts through the *CRZ1*/*TCN1*-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:**3432–3444.
61. **Terashima, H., N. Yabuki, M. Arisawa, K. Hamada, and K. Kitada.** 2000. Up-regulation of genes encoding glycosylphosphatidylinositol (GPI)-attached proteins in response to cell wall damage caused by disruption of *FKS1* in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **264:**64–74.
62. **Tong, A. H., G. Lesage, G. D. Bader, H. Ding, H. Xu, X. Xin, J. Young, G. F. Berriz, R. L. Brost, M. Chang, Y. Chen, X. Cheng, G. Chua, H. Friesen, D. S. Goldberg, J. Haynes, C. Humphries, G. He, S. Hussein, L. Ke, N. Krogan, Z. Li, J. N. Levinson, H. Lu, P. Menard, C. Munyana, A. B. Parsons, O. Ryan, R. Tonikian, T. Roberts, A.-M. Sdicu, J. Shapiro, B. Sheikh, B. Suter, S. L. Wong, L. V. Zhang, H. Zhu, C. G. Burd, S. Munro, C. Sander, J. Rine, J. Greenblatt, M. Peter, A. Bretscher, G. Bell, F. P. Roth, G. W. Brown, B. Andrews, H. Bussey, and C. Boone.** 2004. Global mapping of the yeast genetic interaction network. Science **303:**808–813.
63. **Valvidia, R. H., and R. Schekman.** 2003. The yeasts Rho1p and Pkc1p regulate the transport of chitin synthase III (Chs3p) from internal stores to the plasma membrane. Proc. Natl. Acad. Sci. USA **100:**10287–10292.
64. **Vink, E., R. J. Rodriguez-Suarez, M. Gerard-Vincent, J. C. Ribas, H. de Nobel, H. van den Ende, A. Duran, F. M. Klis, and H. Bussey.** 2004. An *in vitro* assay for (1–6)-β-D-glucan synthesis in *Saccharomyces cerevisiae*. Yeast **21:**1121–1131.
65. **Weiss, E. L., C. Kurischko, C. Zhang, K. Shokat, D. G. Drubin, and F. C. Luca.** 2002. The *Saccharomyces cerevisiae* Mob2p-Cbk1p kinase complex promotes polarized growth and acts with the mitotic exit network to facilitate daughter cell-specific localization of Ace2p transcription factor. J. Cell Biol. **158:**885–900.
66. **Zhang, W., D. Espinoza, V. Hines, M. Innis, P. Mehta, and D. L. Miller.** 1997. Characterization of β-amyloid peptide precursor processing by the yeast Yap3 and Mkc7 proteases. Biochim. Biophys. Acta **1359:**110–122.
67. **Zhao, C., U. S. Jung, P. Garrett-Engele, T. Roe, M. S. Cyert, and D. E. Levin.** 1998. Temperature-induced expression of yeast *FKS2* is under the dual control of protein kinase C and calcineurin. Mol. Cell. Biol. **18:**1013–1022.