Glycosylphosphatidylinositol-anchored Glucanosyltransferases Play an Active Role in the Biosynthesis of the Fungal Cell Wall*

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A novel 1,3-β-glucanosyltransferase isolated from the cell wall of Aspergillus fumigatus was recently characterized. This enzyme splits internally a 1,3-β-glucan molecule and transfers the newly generated reducing end to the non-reducing end of another 1,3-β-glucan molecule forming a 1,3-β linkage, resulting in the elongation of 1,3-β-glucan chains. The GEL1 gene encoding this enzyme was cloned and sequenced. The predicted amino acid sequence of Gel1p was homologous to several yeast zyme was cloned and sequenced. The predicted amino acid sequence of Gel1p was homologous to several yeast genes of Saccharomyces cerevisiae, which encode the putative catalytic subunit of 1,3-β-glucan synthase, have been identified in Aspergillus nidulans (6) and in A. fumigatus.1 However, 1,3-β-glucan chains produced by the 1,3-β-glucan synthase complex remain unorganized and alkali-soluble until covalent linkages occur between 1,3-β-glucans and other cell wall components.

In a search for periplasmic transglycosidases responsible for linking glucans to other cell wall molecules, a newly described 1,3-β-glucanosyltransferase has been identified in A. fumigatus (7). It was isolated from a cell wall autolysate as a 49-kDa polypeptide. The enzyme acts first as an endoglucanase and then transfers the newly generated reducing end to the non-reducing end of another laminarioligosaccharide forming a new 1,3-β linkage. In this study, we report the cloning and the sequencing of the GEL1 (for glucan elongating glucosyltransferase) gene. GEL1 encodes a glycosylphosphatidylinositol (GPI)-anchored protein that is homologous to several yeast proteins such as Gas1p of S. cerevisiae (8–11) or Phrp of Candida albicans (12, 13). GAS1, PHR1, and PHR2 gene products are required for correct morphogenesis in yeast and were so far endowed with an unknown biochemical function. Here we show that Gas1p, Phr1p, and Phr2p display the same 1,3-β-glucanosyltransferase activity as Gel1p of A. fumigatus.

EXPERIMENTAL PROCEDURES

Strains and Standard Growth Conditions—A. fumigatus strain CBS 144.89 was grown in Sabouraud liquid medium (2% glucose + 1% mycopenote, Biokar, Beauvais, France). The S. cerevisiae haploid strain WB2d (gas1::LEU2), generated from the wild-type strain W303-1B (MATa ade2-1 his3-11, 15 trp1-1 ura3-1 leu2-3, 112 can1-100) by one-step disruption (11), was the host strain for complementation experiments. The strain of S. cerevisiae ΔΔ-Esg214, devoid of the major exoglucanase activity, kindly provided by F. Del Rey was used to detect the presence of the 1,3-β-glucanosyltransferase activity in the S. cerevisiae membranes. The S. cerevisiae strains and the wild-type C. albicans CAI-1 (12) were grown on yeast nitrogen base (0.67%) medium containing 2% glucose, 0.5% casamino acids, and the required supplements at 50 mg/ml (YNB) or in YPD medium (1% yeast extract, 2% mycopenote, 2% glucose). Cultures were performed in flats incubated at 25 °C at 200 rpm or in fermenters for 24 h at 25 °C, 500 rpm (7, 15). Escherichia coli JM 101 (Δ (lac pro AB) thi strA supE endA sbeB hsdR F’ (traD36 proAB lacY1 lacZAM15)) was the host strain for recombinant DNA manipulations.

Cloning Procedures and DNA Manipulations—A λEMBL3 genomic library of A. fumigatus was screened with [γ-32P]ATP-labeled degenerated oligonucleotides deduced from the amino acid sequencing of the

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1 A. Beauvais, unpublished observations.
2 The abbreviations used are: GPI, glycosylphosphatidylinositol; PLC, phospholipase C; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; kb, kilobase pair; CRD, cross-reactive determinant; HPAEC, high performance anion exchange chromatography.
NH-terminal and internal peptides obtained after endolysin digestion of the 49-kDa polypeptide previously purified (7). Cloning and sequencing procedures were as described previously (15). The position of the introns was determined after amplification of cDNA by PCR using primers deduced from the genomic DNA sequence. The samples in a 100-μl reaction containing 200 ng of cDNA, 1 μM each primer, 1 ng of cDNA, and 1 unit of Taq polymerase (Amersham Pharmacia Biotech) were subjected to 30 cycles of amplification consisting of the following steps: 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C. The PCR products were subcloned in PCR2.1 (TA Cloning kit, Invitrogen), and sequencing was performed as described previously (15).

**RNA Extraction and Reverse Transcription (RT)-PCR**—RNA Extraction and Reverse Transcription (RT)-PCR was always included as a negative control to check for the presence of contaminating DNA. The cDNA products were then employed as target DNAs for amplifications as described previously using two sets of primers as follows: PGEla 5′-CCCTCGTCTGTCCTCCATG-3′; PGElb 5′-TTGGTGGTGCACGAGCC-3′ for amplification of a 0.35-kb fragment of the GELI gene and Pactin 1 5′-GTGATGGACGACCGACTAC-3′; Pactin 2 5′-GGGGACGACGCTGGTAAAAGC-3′ for amplification of the 5′ region of the Pactin gene (16). The RT-PCR products were resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide for photography.

**Complementation of Δgas1 of S. cerevisiae by A. fumigatus GELI**—A fragment of the GASI gene that contains the promoter region, the start codon, and the signal sequence (11) was amplified by PCR using a sequence-specific 5′ primer containing a HindIII site (underlined), 5′-ACTCAAGCTTATGCTTTGCGAAGCC-3′, and a 3′ primer with a SmaI site (underlined) after the last codon of the signal sequence (at nucleotide +68), 5′-AATCCGCGCAGGTTGCGGACCCGCAA-3′. The resulting PCR product was subsequently digested with HindIII and SmaI and cloned into a HindIII/SmaI-cut pGem-7Zf (+) (Promega Corp., Madison, WI) to give plasmid pHSP. The coding region of the GELI cDNA lacking the NH-terminal was PCR-amplified by the forward primer 5′-CAGAATTCGGACGCTTACCTCCATCA-3′ and the reverse primer 5′-ACTCTAGATCAAGAAGAGCCAGGACCGAAGCAC-3′ with a SmaI site (underlined) after the stop codon, generating a fragment of approximately 1.3 kb with an EcoRI site (underlined) introduced at nucleotide +73 (corresponding to alanine 25) to facilitate the fusion with the 5′ region of the GASI gene. The resulting PCR product was subsequently digested with EcoRI (filled with Klenow) and XhoI and introduced into plasmid pHSP cut with SmaI and XhoI. DNA sequencing confirmed the desired in-frame fusion. The GAS1/GELI fusion was then cloned in the high copy number vector YEp24 and the resulting plasmid used to transform the WB2d strain. Transformation of S. cerevisiae was carried out by the lithium acetate procedure (17). Ura- transformants were selected on uracil-free minimal medium. Membrane proteins were extracted by a modification of the method of Bordier (20). Briefly, 10 μl of membrane extract was incubated with 5 μl of GPI-PLC and at least 10 volumes of buffer at 37 °C for 30 min to 4 h. Buffers were, respectively, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.1% Triton X-114 for the Trypanosoma brucei enzyme and 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 100 mM KCl, 1 mM EDTA, 0.2% Triton X-114, and 20% glycerol for the Bacillus thuringiensis enzyme. GPI-PLC treatment was performed on intact or methanol (4 volumes)-denatured protein extract. Partitioning of membrane proteins with Triton X-114 was done by a modification of the method of Bordier (21). Two antisera used for Western blotting were directed against the cross-reactive determinant (CRD) specific for GPI proteins (Oxford Glycosystems) or Gel1p. Two immunization protocols were used to produce the antiserum to act as an antigen. First, 20 μg of the 49-kDa polypeptide purified from the cell wall autolysate were mixed in 0.9% NaCl (w/v) with an equal volume of Freund’s complete adjuvant and injected intradermally at multiple sites in female Balb/c mice. Two booster injections of the transferrin in the incomplete adjuvant (1:1 (v/v)) were administered at 2-week intervals. Alternatively, rabbits were immunized against a peptide INRKPESYNDYVC described on the basis of sequence conservation (22). A peptide was synthesized with the addition of cysteine to its maleimido-benzoyl-N-hydroxy-succinimide ester, immunization of the animal, and titer determination of the antiserum were performed by Eu-rogentec (Seraing, Belgium). Immunopurification of the specific antipeptide antibodies was done after coupling the peptide to epoxy-activated Sepharose (Amersham Pharmacia Biotech) following the instructions of the manufacturer. Immunoblotting of blots was done using the ECL Western blotting detection procedure of Amersham Pharmacia Biotech.

**Expression of Gas1p in S. cerevisiae**—The production of Gas1p, which is a truncated form of Gas1p lacking the proposed GPI attachment site (Asn528), was induced. The COOH-terminal hydrophobic domain, was expressed by using plasmid pST20, Gas1p528, which was constructed by PCR amplification of the gene with the forward primer 5′-TAGATACCGACGCGCCTGCAA-3′ and the reverse primer 1, 5′-CTAGATCTCAAGAAGACCGACGCGGTTCCTCATTCTAG-3′ and the reverse primer 2, 5′-CTAGATCTCCTAAGCCGCTCTGTGGAAAGCTG-3′ (for the full GELI), or the reverse primer 2, 5′-CTAGATCTCCTAAGCCGCTCTGTGGAAAGCTG-3′ (for the truncated form). The forward primer was complementary to nucleotides +58 to +99 that incorporated an Xhol site (underlined) at the 5′ end. The reverse primer was complementary to nucleotides 1339–1356 of the coding region, encompassing the codon form Ala447 to Leu452, and the reverse primer 2 was complementary to nucleotides 1327 to 1357 of the coding region, encompassing the codon form Ser413 to Ala419. It incorporated an XhoI site (underlined) at the 3′ end. Thirty cycles consisting of 1 min at 95 °C melting step, a 1-min 60 °C annealing step, and 1-min 70 °C extension were run. The resulting PCR products were digested by Xhol and BamHI and cloned into the expression vector pKJ113 digested by the same enzymes, generating the plasmid pISAB1 and the plasmid pISAB2. P. pastoris spheroplasts were transformed with 10 μg of pISAB1 or 10 μg of pISAB2 linearized by EcoRI. Transformants were selected on histidine-deficient medium and screened on minimal methanol plates for insertion of the construct in the P. pastoris GS115 genome as described previously (19). Production of r-Gel1p1,528 and r-Gel1p528 was carried out in recombinant P. pastoris GS115 containing pISAB1 and pISAB2. 10 μg/ml methanol was added to the culture medium, and recombinant protein was purified from the culture filtrate. The production of r-Gel1p1,528 was confirmed by Western blotting with the antibody precipitated with protein A-Sepharose. The production of r-Gel1p528 was confirmed by Western blotting with the antibody precipitated with protein A-Sepharose.
Fig. 1. Comparison of the predicted amino acid sequences of Gel1p of *A. fumigatus* with Gas1p and homologs in *S. cerevisiae*, Phr1p and Phr2p of *C. albicans*, Epd1p and Epd2p in *C. maltosa*, and P78785 and O13692 in *S. pombe*. Identical residues are indicated.
RESULTS

Isolation and Sequence Analysis of the GEL1 Gene Encoding the 1,3-β-Glucanosyltransferase Gel1p—The amino acid sequences of the NH2-terminal peptide and of one internal peptide obtained from the 49-kDa polypeptide (p49) isolated from the cell wall autolysate (7) were DDVPTPTVKGNAFFKDERFY and DAPNVDNDALPAI, respectively, and were used to design two degenerated oligonucleotide probes (probe A, AAGGGYAYGCTCTTAYAAGGYGAAGCGGCTTTCA (KGNAFFKDERFY); probe B, TCRTTTCADCRTCCCACTT (NWDVDND)). Screening of a AEMBL 3a genomic library of A. fumigatus with probe A identified three positive clones. Restriction enzyme analysis of purified bacteriophage DNA revealed that the three clones had a common 2.2-kb XbaI fragment that hybridized with both probes A and B.

The 2.2-kb genomic DNA fragment contained the entire open reading frame of the GEL1 gene. The primers P1 (5’-GACGAGCTTACTCCCATCCTACT-3’) and P2 (5’-GGGTATGAGAAGAAATCA-3’), deduced from the genomic DNA sequence, were used to clone the corresponding cDNA by PCR. Analysis of the sequences of the complementary and genomic DNA showed that the gene was 1356 nucleotides long and contained an open reading frame predicting a 452-residue polypeptide with a theoretical molecular mass of 44 kDa (Fig. 1). GEL1 gene contained one intron of 60 base pairs starting before nucleotide 598.

FASTA and BLAST searches of the GenBank™ and EMBL data banks showed significant homologies of Gel1p with a family of GPI-anchored proteins (Gas1p to Gas5p of S. cerevisiae, Phr1p and Phr2p of C. albicans, and Epd1p and Epd2p of Candida maltosa (with 37, 34, 37, 36, 38, 33, and 35% identity, respectively) playing a role in yeast morphogenesis (8–13, 25, 26) and with two uncharacterized sequences of Schizosaccharomyces pombe found in data base (P78785 and O13692 with 45 and 35% identity, respectively) (Fig. 1). Gel1p was shorter than the other proteins, and the highest homology was seen in the first 325 amino acids. The position of the first 6 of the 13 cysteines was conserved between Gel1p and all the other homologous yeast proteins. Several significant features are conserved among all these proteins as follows: (i) a hydrophobic amino terminus characteristic of secretory signal sequences, (ii) several putative N-glycosylation sites, (iii) a COOH-terminal region rich in serine residues that are potential sites for O-glycosylation, and (iv) a hydrophobic carboxyl terminus characteristic of GPI-anchored proteins.

The predicted protein Gel1p had both hydrophobic amino and carboxyl termini, 17 and 26 amino acids long, respectively. The signal peptidase cleavage site according to the consensus (3, 4) rule (27) was Ala19 for Gel1p, and the ω, ω + 1, and ω + 2 site for GPI attachment based on the consensus predicted cleavage of GPI anchor (28, 29) was Gly418, Ala419, and Ala420 (Fig. 1). Three records consensus N-glycosylation sites were found located at amino acid residues 249, 329, and 337, in agreement with previously published biochemical data showing that Gel1p was N-glycosylated (7).

RT-PCR data and Western blot analysis showed that Gel1p is constitutively expressed, during exponential and linear growth (up to 48 h in our culture conditions). In contrast to PHR, the expression of GEL1 is not pH-regulated since a similar expression level was seen at pH 4 and 8 (data not shown).

Biochemical Data Confirmed That Gel1p Is GPI-anchored—Antisera were directed either against the entire p49 or one of its immunogenic peptides, labeled by Western blot, a protein with an apparent mass of 54 kDa in a membrane extract (data by boxes). The amino and carboxyl hydrophobic termini of Gel1p were underlined. The putative cleavage site of secretory signal sequence and the putative GPI attachment site are indicated by an arrow. The three potential N-glycosylation sites of Gel1p are indicated by an asterisk. The peptide sequence used as probe to screen the genomic library is underlined.
GPI-anchored Glucanosyltransferase Involved in Cell Wall Synthesis

not shown). Gel1p was recovered in the Triton X-114 fraction of a membrane extract indicating that it was an integral membrane protein. Incubation of this extract with GPI-PLC resulted in the recovery of Gel1p in the aqueous phase (Fig. 2A). This band was also positive with the anti-CRD antibody which is specific for a cyclic phosphate formed after the cleavage of the GPI anchor by GPI-PLC (data not shown). All attempts to radiolabel Gel1p using [3H]inositol or [14C]ethanolamine have failed thus preventing the biochemical isolation of the peptide tail bound to GPI (data not shown). To further confirmation on the GPI-anchoring of Gel1p and particularly to identify the ω sites for GPI-attachment, GEL1 (Gel1p452) and a truncated form of GEL1 lacking the carboxyl terminus (Gel1p419) were expressed in P. pastoris. Western blot analysis using anti-Gel1p antibodies showed that (i) Gel1p419 was recovered in the culture filtrate and (ii) Gel1p452 was found in the Triton X-114 fraction of a membrane extract from P. pastoris (Fig. 2B). Treatment of this detergent extract with GPI-PLC resulted in the release of Gel1p452 in the aqueous phase (Fig. 2B) associated to a CRD positivity of the GPI-PLC cleaved Gel1p. Peptide cleavage may result from a proteolytic degradation. In addition, p49 reacted negatively with the anti-CRD antibody suggesting that a peptide cleavage of Gel1p has also occurred at the COOH terminus during autolysis. p49 is a hydrophilic, C- and N-truncated form of Gel1p. Peptide cleavage may result from proteolytic degradation occurring during the biochemical purification of the protein but may also be an alternative for the differential regulation of Gel1p anchoring to the membrane.

Gas1p, Phr1p, and Phr2p Share the Same Enzymatic Activity as Gel1p—The sequence homologies between Gel1p, Gas1p, Phr1p, and Phr2p suggested that all these proteins shared the same biochemical function. Identification of the conserved regions in the sequences of the homologous proteins and the previous discovery of the glucanosyltransferase activity in a truncated hydrophilic Gel1p polypeptide suggested that the COOH and NH2 termini were not essential for the enzymatic activity. For this reason, the putative enzymatic activity of the Gas1p, Phr1p, and Phr2p was compared with the one of Gel1p using recombinant proteins expressed without a GPI attachment signal (Gel1p419) and was therefore secreted in the medium. These proteins were purified as polypeptides with mass of 60, 76, 94, and 125 kDa for Gel1p, Phr1p, Phr2p, and Gas1p, respectively (Fig. 3).

Analysis by HPAEC of the product resulting from the incubation of recombinant proteins with reduced laminaranidecose is shown in Fig. 4. Fig. 4A shows the enzymatic kinetics obtained with the recombinant Gel1p. After 1 h incubation, major initial products were rG6, rG7, rG8, rG18, rG19, and rG20 in agreement with the two-step reaction scheme previously described by Hartland et al. (7) (E + rG13 → E.G5 + rG8 + E.G6 + rG7 + E.G7 + rG6; E.G5 + E.G6 + E.G7 + rG13 → E + rG18 + rG19 + rG20) (E corresponds to the enzyme Gel1p, and the oligosaccharides in bold are the reaction products). HPAEC data indicated that all products contained 1,3-β linkages since the introduction of a linkage different from 1,3-β-glucosidic linkage will result in a shift in the retention time of the branched oligosaccharide (15, 30). Longer incubation time (8–20 h) with a 3 mM substrate concentration resulted in the production of a range of oligosaccharides with a degree of polymerization from 5 to 40 (Fig. 4A). The complex HPAEC pattern seen with prolonged incubation showed that the initial transferase products can be reused subsequently either as donors or acceptors resulting in a wide range of transfer products with increasing size (degree of polymerization >30) until they become alkali-insoluble. Analysis of the products resulting from the incubation of recombinant protein
Gas1p, Phr1p, and Phr2p with reduced laminaritridecaose showed an HPAEC pattern identical to the one obtained with the recombinant protein Gel1p, characterized by the sole presence of laminarioligosaccharides (Fig. 4B). Consequently, Gas1p, Phr1p, and Phr2p displayed a 1,3-β-glucanosyltransferase activity similar to the one characterized for Gel1p.

To determine the minimal size of the oligosaccharide used in the transfer reaction, which promotes the release of one oligosaccharide from the reducing end and the production of an unique transfer product, recombinant proteins were incubated with 3 mM reduced laminarioligosaccharide containing 13 glucose units (G13) in 20 μl of 50 mM NaOAc, pH 5.5, at 37 °C. A 2.5-μl aliquot supplemented with 40 μl of 50 mM NaOH was analyzed by high pressure liquid chromatography with a CarboPAC PA-1 column and a pulsed electrochemical detector. A, analysis of product after 0, 1, 8, and 20 h of incubation with Gel1p. B, analysis of products obtained with Gas1p, Phr1p, and Phr2p (Tn, identical to A).

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Gel1p and Homologous Yeast Proteins Are Functional in Vivo—A similar 1,3-β-glucanosyltransferase activity has been demonstrated for recombinant GPI-truncated Gel, Gas, and Phr proteins. It was then essential to verify that the native GPI-anchored proteins were functional. For this purpose, membrane extracts were incubated with reduced laminarioligosaccharide (G13 and G14). HPAEC analysis showed that membrane extracts displayed a 1,3-β-glucanosyltransferase activity. This activity was detected with membrane extracts from A. fumigatus, C. albicans, and S. cerevisiae (data not shown).

To gain further insight on the functionality of Gel1p in vivo, the GEL1 cDNA was expressed in a gas1 strain (WB2d) of S. cerevisiae. A major band of about 62 kDa was detected by anti-Gel1p antiserum in total extracts of yeast clones transformed with GEL1 under the control of the GAS1 promoter (Fig. 5A). The absence of this band in the Δgas1 strain indicated that the 62-kDa polypeptide corresponded to the GEL1 gene product. The lower mobility of the recombinant protein compared with the native A. fumigatus Gel1p (54 kDa) suggested a higher degree of mannosylation of the protein expressed in S. cerevisiae. A similar modification was seen when Gel1p was expressed in P. pastoris (Fig. 2B). A null mutation in the GAS1 gene caused several morphogenetic defects as follows: cells had an abnormal morphology, became round and larger, and were defective in bud maturation and cell separation, assuming a clumped aspect in stationary phase. The cells were more sensitive to Calcofluor White and were more resistant to zymolyase (9). GEL1 was able to rescue the morphological defects of the mutant Δgas1; upon microscopic analysis, transformed cells showed a normal ellipsoidal shape, and in stationary phase very few clumped cells were detected (data not shown). As shown in Fig. 5B, GEL1 almost completely abolished the Δgas1 hypersensitivity to growth in the presence of Calcofluor White. Complementation of the Δgas1 mutant by...
GEL1 resulted in a decrease of fluorescence consecutive to Calcofluor White staining (data not shown), suggesting a decrease in the amount of chitin brought about by the lack of Gas1p (31). To confirm this observation, the chitin level in exponentially growing cells has been quantified in the zymolyase-undigestible pellet of the alkali-insoluble fraction. In Δgas1 cells harboring GEL1 cDNA, the level of glucosamine reached 1.4% (w/w of the pellet) indicating a reduction of the increase of chitin from 7 to ~3-fold with respect to Δgas1 control cells. In addition, the ratio of total hexose concentration of the alkali-soluble/alkali-insoluble fractions was reduced in the WB2d strain harboring GEL1 cDNA in a trend similar to the WB2d strain complemented with the GAS1 gene (data not shown). Another phenotypic trait that was considered has been the sensitivity of intact cells to zymolyase treatment. After 45 min of incubation at 30 °C with zymolyase 100T (12.5 units/ml), Δgas1 cells were almost completely resistant to the enzyme, as previously reported (9). The wild-type strain was very sensitive with a 85% decrease in A660 nm, whereas the cells expressing Gel1p showed a high (although intermediate) sensitivity with a 60% decrease. Altogether, these data indicate that Gel1p significantly reduced both the defects of gas1 mutant and the compensatory responses induced by the lack of Gas1p in S. cerevisiae, confirming that Gel1p is functional in S. cerevisiae and that these two proteins display similar enzymatic function both in vivo and in vitro.

**DISCUSSION**

GEL1, which encodes a 1,3-β-glucanosyltransferase previously identified in A. fumigatus (7), is homologous to GAS/PHR/EPD genes encoding a family of GPI-anchored proteins required for correct morphogenesis in yeast. In Candida, PHR and EPD are involved in conducting apical growth since null mutants are affected in the formation of germ tubes or pseudohyphal growth (12, 13, 25). Their biochemical function was unknown until this study. We have now demonstrated that Gel1p, Phr1p, Phr2p, and Gas1p display the same 1,3-β-glucanosyltransferase activity responsible for elongation of 1,3-β-glucans. In addition, complementation experiments have shown that the genes of these different families encode structurally and functionally related proteins as follows. (i) PHR1 and GEL1 can complement Δgas1 mutation in S. cerevisiae (Ref. 18 and this study). (ii) Engineered expression of PHR1 in a Δphr2 mutant strain and PHR2 in Δphr1 mutant strain complement the defects in the opposing mutant (12). These complementation studies have also confirmed the absence of a functional role of the COOH-terminal serine/threonine stretch and O-glycosylation in the enzymatic activity, as it was already shown for Gas1p (22).

Although their enzymatic activity is common, the regulation of the expression of the different encoding genes can be under the control of different signals. In Candida, the pH and the nutritional composition of the culture medium play a major role (12, 13, 25). In C. albicans, a differential role of the pH was not due to a different pH optimum of the enzymatic activity of Phr1p and Phr2p since both recombinant proteins presented an acidic pH optimum (around 5) and both were inactive at pH 7.5 after 8 h incubation (data not shown). In S. cerevisiae and in A. fumigatus, expression of GAS1 and GEL1 seems constitutive (Refs. 10, 32, and this study).

In S. cerevisiae, Δgas1 mutant has a reduced growth rate that becomes more severe at neutral pH and is characterized by a high percentage of budded cells at stationary phase. Several biochemical arguments indicate that the organization of the cell wall is altered including the following: (i) a decrease in the glucan content and a modification of its structure suggested by differences in the ratio of 1,3/1,6-β-glucan linkages and alkali solubility of cell wall fractions; (ii) a release of 1,3-β-glucan and/or β-glucoylated cell wall proteins in the culture medium; (iii) an increase of chitin level; (iv) an increased incorporation of cell wall mannoproteins, specifically of Cwp1p, which become cross-linked to chitin instead of glucans (31, 33).

In C. albicans, deletion of PHR genes resulted in pH-conditional defects in growth, morphogenesis, and virulence (12, 13, 34). PHR1 is expressed at neutral to alkaline pH. At alkaline pH, the Δphr1 mutant is not able to conduct apical growth of either yeast or hyphal growth forms. Cells become larger and ronder, a phenotype reminiscent of the Δgas1 mutant. It was shown that the phenotypic defects of the mutants were not associated with defective cytoskeletal polarization or secretion, suggesting that PHR1 was involved in cell wall organization (13). Cell wall analysis of the Δphr1 mutant showed a doubling in the ratio between the alkali-soluble and -insoluble glucans, an increase in the chitin level, and a substantial reduction in 1,6-β-glucan (35). As the Δgas1 mutant, Δphr1 mutant is hypersensitive to Calcofluor White and to Nickkomycin Z.

In contrast to PHR1, PHR2 is only expressed at acidic pH (pH 5) (12). A Δphr2 mutant manifests pH-conditional defects in growth analogous to those of a PHR1 mutation but at acidic pH rather than alkaline pH values. The mutant exhibits reduced growth at pH 6. Arrest of growth is associated with an isotropic enlargement of the cells and altered bud morphology, but yeast remains viable at the restrictive pH. Analysis of the cell wall...
has not been performed for αPrh2 mutant.

In C. maltosa, a ∆epd1 mutant showed a reduced growth rate at pH 4 with morphological defects of the cells (large and round yeast with multiple buds) similar to ∆gas1 mutant (9, 25). At pH 7, no morphological differences were noted. Transition of yeast to pseudohyphal growth was abolished in the ∆epd1 mutant at pH 4 but not at pH 7. This pattern is somehow reminiscent of ∆Prh2 mutant except that ∆epd1 mutant grows at pH 4, whereas ∆prh2 does not. The cell wall of the ∆epd1 mutant is characterized by an increase in chitin and a decrease in 1,6-β-glucans. However, in contrast to αPrh1 and ∆gas1 cells, the levels of both alkali-soluble and alkali-insoluble glucan fractions were reduced (25).

Recent data obtained in the chemical characterization of the structural polysaccharides of the cell wall of A. fumigatus and previous studies on the cell wall of S. cerevisiae as follows: (i) the reduction of the 1,3-β-glucans (ii) the secretion in the culture medium of β-glucan side chains with free non-reducing ends of 1,3-β-glucans; (ii) the secretion in the culture medium of β-glucan side chains (step iii in the biosynthetic pathway). By doing so, it would increase the

acknowledged proteins without known enzymatic function (like Ags1, Cwp1p, or Cwp2p) are covertly incorporated to the cell wall after cleavage of the GPI anchor and become part of three-dimensional network composed by cell wall polymers (36, 42, 43).

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