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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 protein families encoded by *GAS* of *Saccharomyces cerevisiae*, *PHR* of *Candida albicans*, and *EPD* of *Candida maltosa*. Although the expression of these genes is required for correct morphogenesis in yeast, the biochemical function of the encoded proteins was unknown. The biochemical assays performed on purified recombinant Gas1p, Phr1p, and Phr2p showed that these proteins have a 1,3-β-glucanosyltransferase activity similar to that of Gel1p. Biochemical data and sequence analysis have shown that Gel1p is attached to the membrane through a glycosylphosphatidylinositol in a similar manner as the yeast homologous proteins. The activity has been also detected in membrane preparations, showing that this 1,3-β-glucanosyltransferase is indeed active *in vivo*. Our results show that transglycosidases anchored to the plasma membrane via glycosylphosphatidylinositols can play an active role in fungal cell wall synthesis.

The cell wall of the human opportunistic fungal pathogen *Aspergillus fumigatus* is a complex structure mainly composed of polysaccharides, 1,3-β-glucan being the most abundant (1, 2). In a way similar to other fungi, 1,3-β-glucans of *A. fumigatus* serve as a skeleton on which the other polysaccharides of the cell wall (chitin and galactomannan) become anchored (3). In filamentous fungi and in yeast, 1,3-β-glucans are synthesized by a plasma membrane-bound glucan synthase complex, which uses UDP-glucose as a substrate and extrudes 1,3-β-glucan chains through the membrane into the periplasmic space (4, 5). Genes homologous to the *FKS* 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 glucanosyltransferase) 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% mycopeptone, Biokar, Beauvais, France). The *S. cerevisiae* haploid strain WB2d (*gas1::LEU2*), generated from the wild-type strain W303-1B (*MATα 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* Δ*Exg2* (14), 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* CAF3-1 (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% mycopeptone, 2% glucose). Cultures were performed in flasks 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 sbcB hsdR F'* (*traD36 proAB lacI*^{q} *lacZΔM15*)) was the host strain for recombinant DNA manipulations.

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

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF072700.

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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_2-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 volume containing 200 μM each of dNTP, 100 pmol of 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 was isolated from mycelium of *A. fumigatus* grown in Sabouraud liquid culture using a Qiagen RNA/DNA kit. Reverse transcription was carried out with the Promega Reverse Transcription System kit following the instructions of the manufacturer. A tube containing all the reaction components and heat-inactivated (10 min at 94 °C) avian myeloblastosis virus-RT 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: PGel1a 5′-CCTCTGCTGCTCCCTACG-3′; PGel1b 5′-GTTGGTGTTGCAGCC-3′ for amplification of a 0.35-kb fragment of the *GEL1* gene and Pactin 1 5′-GGTGATGAGCCACAGT-CCAAG-3′; Pactin 2 5′-GGGGACGACGTGGGTAACAAC-3′ for amplification of a 0.3-kb fragment of the actin gene as a control deduced from the *A. nidulans* 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 GEL1—A fragment of the *GAS1* 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 *Hin*dIII site (underlined), 5′-ACTCAAGCTTATCGATTACTGGCATACAATGGT-3′, and a 3′ primer with a *Sma*I site (underlined) after the last codon of the signal sequence (at nucleotide +68), 5′-AATCCCGGGCAGTTGCGACGCCAGCAAA-3′. The resulting PCR product of approximately 1 kb was then digested with *Hin*dIII and *Sma*I and cloned into a *Hin*dIII/*Sma*I-cut pGEM-7Zf(+) (Promega Corp., Madison, WI) to give plasmid pHSP. The coding region of the *GEL1* cDNA lacking the NH_2 terminus was PCR-amplified by the forward primer 5′-CAGGAATTCGACGACGTTACTCCCATC-A-3′ and the reverse primer 5′-ACTCTAGAATCCAAGAGGACGAGG-CCAGC-3′ with the *Xba*I site (underlined) after the stop codon, generating a fragment of about 1.3 kb with an *Eco*RI site (underlined) introduced at nucleotide +73 (corresponding to alanine 25) to facilitate the fusion with the 5′ region of the *GAS1* gene. The resulting PCR product was subsequently digested with *Eco*RI (filled with Klenow) and *Xba*I and introduced into plasmid pHSP cut with *Sma*I and *Xba*I. DNA sequencing confirmed the desired in frame fusion. The *GAS1*/*GEL1* 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* cells was carried out by the lithium acetate procedure (17), and transformants were selected on YNB without amino acid, 2% glucose, and auxotrophic supplements. The transformed strain is indicated throughout the text as WB2d (YEp-*ScGEL1*). Other strains harboring plasmids used were WB2d (YEp-*GAS1*) (18) and WB2d (YEp), obtained after transformation of WB2d with the YEp24 plasmid without insert.

Expression of Gel1p in Pichia pastoris—*P. pastoris* GS115 (Invitrogen) and the expression vector pKJ113 (19) were used to express recombinant *A. fumigatus* Gel1p. The full open reading frame of *GEL1* ($Gel1p_{452}$) and a truncated form of *GEL1* ($Gel1p_{419}$) were generated by PCR amplification of the gene with the forward primer 5′-TATCTCG-AGCCCCCTCCATCAAGGCTCGTGACGACGTTACTCCCATCACT-3′ and the reverse primer 1, 5′-CTAGGATCCTCACAAGAGGACGAGGC-CAGC-3′ (for the full *GEL1*), or the reverse primer 2, 5′-GTAGGATC-CCTAAGCGCCCTTGGAAGAGGTGGA-3′ (for the truncated form). The forward primer was complementary to nucleotides +58 to +99 that incorporated an *Xho*I site (underlined) at the 5′ end. The reverse primer 1 was complementary to nucleotides 1339–1356 of the coding region, encompassing the codon form Ala^{447} to Leu^{452}, and the reverse primer 2 was complementary to nucleotides 1237–1257 of the coding region, encompassing the codon form Ser^{413} to Ala^{419}. It incorporated an in frame TAG stop codon and a *Bam*HI 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 *Xho*I and *Bam*HI 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 *Eco*RI. 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-$Gel1p^{452}$ and r-$Gel1p^{419}$ placed under the control of the alcohol oxidase promoter in *P. pastoris* was obtained consecutively to the addition of 0.7% methanol to the culture medium (Invitrogen manufacturer's instructions).

Biochemical Characterization of Gel1p—To extract membrane proteins of *A. fumigatus* or *P. pastoris* expressing recombinant Gel1p, mycelium or yeast cells were resuspended in a Tris-HCl, pH 7.5, buffer containing 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and disrupted for 3 min with 1-mm diameter glass bead in an MSK (Braun) cell breaker (or 10 min with 0.5-mm diameter glass for yeast cells).

The cell homogenates were then centrifuged for 10 min at 4,000 × *g* to remove the cell walls. The 4,000 × *g* supernatants were centrifuged for 1 h at 36,000 × *g,* and the membrane pellets were suspended in the extraction buffer and stored at −20 °C. GPI-PLC treatment of membrane extracts was performed as recommended by Oxford Glycosystems (Abingdon, UK). 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 100 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol 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 (20). Briefly, 20 μl of a membrane extract was resuspended in 40 volumes of the extraction buffer containing 2% Triton X-114 (Sigma). After 1 h at 4 °C, the suspension was incubated for 30 s at 37 °C. The two phases were separated by a 30-s centrifugation; the aqueous phase was extracted 3 times after adding 0.02 volume of concentrated Triton X-114, and the detergent phase was extracted 3 times with the extraction buffer. Proteins were precipitated with trichloroacetic acid (5–10% final concentration) and washed with cold acetone. SDS-polyacrylamide gel electrophoresis of the different protein extracts was performed on a 10% separating gel with a 4% stacking gel. Electrotransfer of proteins to nitrocellulose membrane (0.2-μm pore size, cellulose nitrate (Schleicher & Schuell)) was done overnight at 30 V in a 50 mM Tris, 200 mM glycine, 20% methanol buffer (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 anti-transferase antiserum. 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 transferase in Freund's incomplete adjuvant (1:1 (v/v)) were administered at 2-week intervals. Alternatively, rabbits were immunized against a peptide INRAKPKESYNDVYC designed on the basis of sequence data. Coupling of the peptide through cysteine to *m*-maleimido-benzoyl-*N*-hydroxy-succinimide ester, immunization of the animal, and titer determination of the antiserum were performed by Eurogentec (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. Immunolabeling of blots was done using the ECL Western blotting detection procedure of Amersham Pharmacia Biotech.

Expression of Gas1p in S. cerevisiae—The production of $Gas1p^{535}$, which is a truncated form of Gas1p lacking the proposed GPI attachment site (Asn^{528}) and the COOH-terminal hydrophobic domain, was done using plasmid pS^{526} gp115 previously described (22). This plasmid, which contains an *Sfu*I site in the second codon upstream from Asn^{528}, was digested with *Sfu*I and *Hin*dIII, filled in, and used to transform the WB2d (*gas1::LEU2*) strain. The transformed strain was grown for 24 h in YNB medium, and the culture filtrate was stored frozen.

Expression of Phr1p and Phr2p in S. cerevisiae—Recombinant Phr1p and Phr2p were expressed from the galactose-inducible promoter of vector pYES2 (Invitrogen). A truncated form of *PHR1* was generated by PCR amplification of the gene with the forward primer 5′-AAGAATT-CCAAACTACAGGTTGAAGCCA-3′ and the reverse primer 5′-GAATT-CTCGAGCTATTTAACTCCAGAGCTTGAGCT-3′. The forward primer was complementary to nucleotides −28 to −8, relative to the translational start codon, and incorporated an *Eco*RI site (underlined) at the 5′ end. The reverse primer was complementary to nucleotides 1540–1560

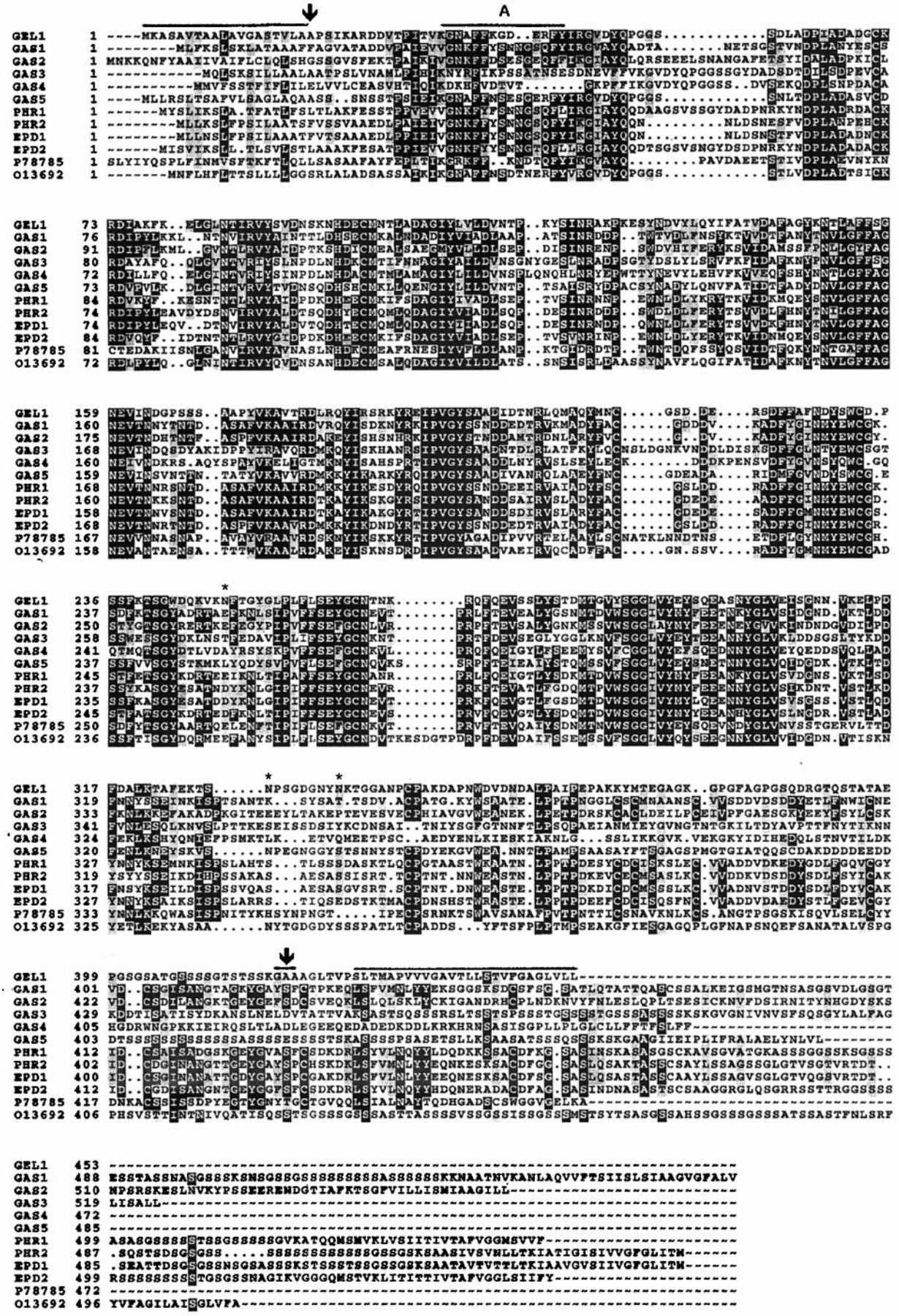

FIG. 1. **Comparison of the predicted amino acid sequences of Gel1p of *A. fumigatus* with Gas1p and homologs in *S. cerevisiae*, Phr1p and Phr2p of *C. albicans*, Epd1p and Epd2p in *C. maltosa*, and P78785 and O13692 in *S. pombe*.** Identical residues are indicated

of the coding region, encompassing the codons for Ser^{514} to Lys^{520}. It incorporated an in frame TAG stop codon adjacent to the Lys^{520} codon and an *Xho*I site (underlined) at the 3′ end. The resulting gene encoded a carboxyl-truncated form of Phr1p which is secreted from the cells due to the absence of the GPI attachment site. This product was directionally cloned into the *Eco*RI and *Xho*I sites of pYES2. A truncated form of *PHR2* was produced in an analogous manner using the forward primer 5′-AAGAATTCATTCGATCGCTATGTTGTTGAA- 3′ and the reverse primer 5′-TTTCTCGAGTTATTATTTACTACCACTTGAACCAGA- 3′. The forward primer was complementary to nucleotides −12 to +11. The reverse primer was complementary to nucleotides 1528–1548 encompassing the codons for Ser^{510} to Lys^{516}. This primer incorporated two in frame TAA termination codons adjacent to the Lys^{516} codon. The plasmids were transformed into *S. cerevisiae* strain INVSC2 (*MATα his3-Δ200 ura3-167*) using a modified lithium acetate method (23) and selecting for Ura^+ transformants. Phr1p and Phr2p were expressed after transfer of a 24-h culture of strains pYES2 *PHR2* and pYES2 *PHR1* to a 2% galactose-based medium.

Enzymatic Analysis of Recombinant Proteins—Culture filtrates containing the recombinant truncated forms of Gas1p, Phr1p, Phr2p, and Gel1p (all lacking the GPI-anchoring COOH terminus) were stored at −20 °C. To assay the enzymatic activity, purification of the recombinant proteins was necessary since endogenous 1,3-β-glucanase and 1,3/1,6-β-glucanosyltransferase activities (15, 24) were always secreted in the culture medium by the yeast heterologous host. Although released in a low amount in comparison to the recombinant protein of interest, their presence would interfere with the determination of the activity since these contaminating enzymes acted on the laminarioligosaccharide substrate or/and reaction products (data not shown).

$Gas1p^{535}$ and $Gel1p^{419}$ were purified with the same chromatographic procedure. After dialysis against a 10 mM Tris-HCl, pH 7, buffer, the culture filtrates were applied to an anion exchange chromatography column of DEAE-5PW (TosoHaas, 8 × 75 mm) equilibrated in the same buffer at a flow rate of 0.7 ml/min. The recombinant proteins were eluted with NaCl gradient (0–250 mM in 50 min and 250–500 mM in 10 min). Phr1p and Phr2p were purified with two chromatographic steps as follows. After dialysis against 10 mM Tris-HCl, pH 7, buffer, the culture filtrates were applied to an anion exchange chromatography column of DEAE-Sepharose Fast Flow (2.4 × 12 cm; Amersham Pharmacia Biotech) equilibrated in the same buffer at a flow rate of 20 ml/h. The recombinant proteins were eluted with a linear gradient of NaCl (0–500 mM, 500 ml). Then fractions containing Phr1p or Phr2p were applied to a gel filtration column of Superdex S-75 (HiLoad 26/60, Amersham Pharmacia Biotech) equilibrated in 10 mM Tris-HCl, pH 7, 200 mM NaCl at a flow rate of 0.4 ml/min. 1,3-β-Glucanosyltransferase activity was analyzed as described previously (7). Briefly, the purified proteins were incubated at the concentrations of 0.05–0.16 mg/ml with 3 mM reduced laminarioligosaccharide of various degrees of polymerization (8 to 14) in a 50 mM acetate buffer, pH 5.5, at 37 °C. Sequential aliquots of 2.5 μl supplemented with 40 μl of 50 mM NaOH were analyzed by HPAEC with a CarboPAC-PA1 column (Dionex 4.6 × 250 mm) as described previously (7).

1,3-β-Glucanosyltransferase Activity of Membrane Extracts—To verify that the 1,3-β-glucanosyltransferase activity was indeed expressed *in situ*, membrane extracts were prepared from *C. albicans*, *A. fumigatus,* and *S. cerevisiae*. Cells were resuspended and disrupted in a 200 mM Tris-HCl, pH 8, buffer containing 50 mM EDTA, 5 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride using an MSK (Braun) cell breaker as described above. The membrane pellet, recovered after two successive 60-min 20,000 × *g* centrifugation steps, was stored at −80 °C in the same buffer. For the assay, total membrane extract (0.5 mg of protein) was resuspended in 50 μl of 100 mM sodium acetate, pH 5.5, buffer containing 0.2% octyl glucoside. 17 μl of membrane suspension were incubated with 2 μl of deoxynojirimycin to inhibit exo-1,3-β-glucanase and 3 μl of 20 mM rG13, at 37 °C for 0, 1, 3, 7 and 20 h. At each time 2.5 μl of mixture were taken, and the enzymatic reaction was stopped with 40 μl of 50 mM NaOH, and products were analyzed by HPAEC using a Carbo PAC PA1 column (Dionex 4.6 × 250 mm) as described previously (7).

RESULTS

Isolation and Sequence Analysis of the GEL1 Gene Encoding the 1,3-β-Glucanosyltransferase Gel1p—The amino acid sequences of the NH_2-terminal peptide and of one internal peptide obtained from the 49-kDa polypeptide (p49) isolated from the cell wall autolysate (7) were DDVTPITVKGNAFFKGDERFY and DAPNWDVDNDALPAI, respectively, and were used to design two degenerated oligonucleotide probes (probe A, AAGGGYAAYGCYTTCTTYAAGGGYGAYGAGCGYTTCTA (KGNAFFKGDERFY); probe B, TCRTTRTCDACRTCCCARTT (NWDVDND)). Screening of a λEMBL 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 *Xba*I 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′ GACGACGTTACTCCCATCACT 3′) and P2 (5′ GGGTATGAGAAGAACAAATCA 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, 49, 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 (−3, −1) rule (27) was Ala^{19} 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 Gly^{418}, Ala^{419}, and Ala^{420} (Fig. 1). Three potential 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 addition, in contrast to *PHR*, the expression of *GEL* 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 are *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*.

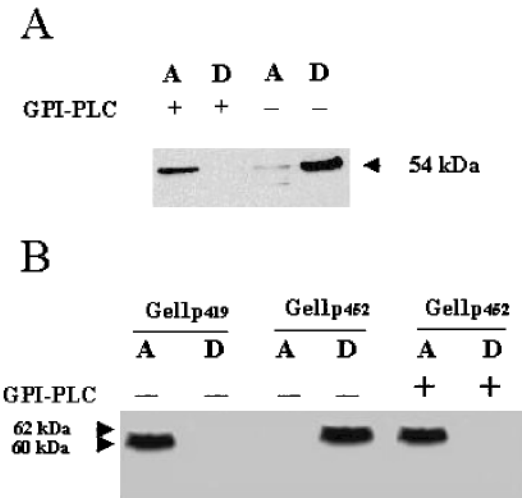

FIG. 2. **Solubilization of membrane-bound Gel1p by GPI-PLC.** *A,* aqueous upper phase (*A*) and detergent lower phase (*D*) after Triton X-114 partition of GPI-PLC treated (+) or control (−); immunolabeling with anti-Gel1p antibody. *B,* Western blot of a Triton X-114 extract of a membrane fraction of *P. pastoris* which expresses Gel1p^{452} (with GPI-anchor) or Gel1p^{419} (without GPI-anchor) in aqueous upper phase (*A*) and in detergent lower phase (*D*) immunolabeled with anti-Gel1p antibody. *1st* and *2nd lanes,* Gel1p^{419}; *3rd* and *4th lanes,* Gel1p^{452}; and *5th* and *6th lanes,* Gel1p$_{452}$ treated by GPI-PLC.

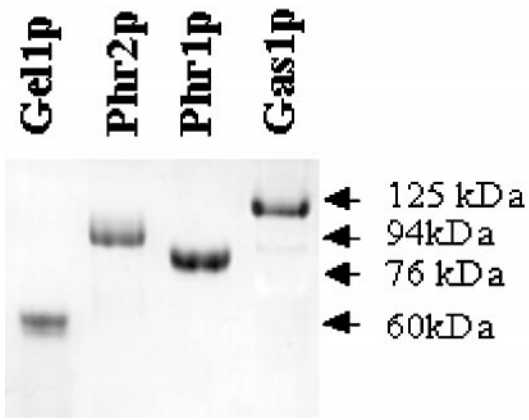

FIG. 3. **SDS-polyacrylamide gel electrophoresis of recombinant proteins Gel1p, Gas1p, Phr1p, and Phr2p stained with Coomassie Blue as described by Neuhoff *et al.* (41).** The mass of each recombinant protein is indicated on the *right.*

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 commercial GPI-PLC resulted in the recovery of Gel1p in the aqueous phase (Fig. 2*A*). 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 [^{14}C]inositol or [^{14}C]ethanolamine have failed thus preventing the biochemical isolation of the peptide tail bound to GPI (data not shown). To get further confirmation on the GPI-anchoring of Gel1p and particularly to identify the ω sites for GPI-attachment, *GEL1* (Gel1p^{452}) and a truncated form of *GEL1* lacking the carboxyl terminus (Gel1p^{419}) were expressed in *P. pastoris*. Western blot analysis using anti-Gel1p antibodies showed that (i) Gel1p^{419} was recovered in the culture filtrate and (ii) Gel1p^{452} was found in the Triton X-114 fraction of a membrane extract from *P. pastoris* (Fig. 2*B*). Treatment of this detergent extract with GPI-PLC resulted in the release of Gel1p^{452} in the aqueous phase (Fig. 2*B*) associated to a CRD positivity of the GPI-PLC cleaved rGel1p^{452} (data not shown). All together, these data showed that Gel1p was bound to the membrane through a GPI anchor and suggested that Gly^{418} was the amino acid responsible for GPI attachment. These data were in agreement with the predictions obtained from the amino acid sequence analysis.

Gel1p either in its membrane-bound or GPI-PLC-cleaved forms migrated at a slightly higher mass (54 kDa) than the p49 hydrophilic polypeptide isolated originally from a cell wall autolysate (7). According to sequence data, the signal peptidase cleavage site should occur after the Ala^{19}, whereas the NH^2-terminal amino acid identified in the biochemically purified p49 polypeptide was Asp^{27}. The exact identity of the NH$_2$-terminal sequence of the Gel1p was investigated with the strain of *P. pastoris* expressing Gel1p^{419}. The coding region of the *GEL1* gene that was integrated in *P. pastoris* started at nucleotide 49 corresponding to Val^{17}. The NH$_2$-terminal amino acid sequence of the recombinant secreted Gel1p obtained was APS. This result confirmed that the signal peptide of Gel1p was indeed composed of 19 amino acids with the NH$_2$-terminal amino acid of the mature Gel1p being Ala^{20} (Fig. 1). The loss of the NH$_2$-terminal peptide APSIKAR from the native protein resulted 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 NH$_2$ 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 (Gel1p^{419}) 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 laminaritridecaose is shown in Fig. 4. Fig. 4*A* 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 only 1,3-β linkages since the introduction of a linkage different from the 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. 4*A*). 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

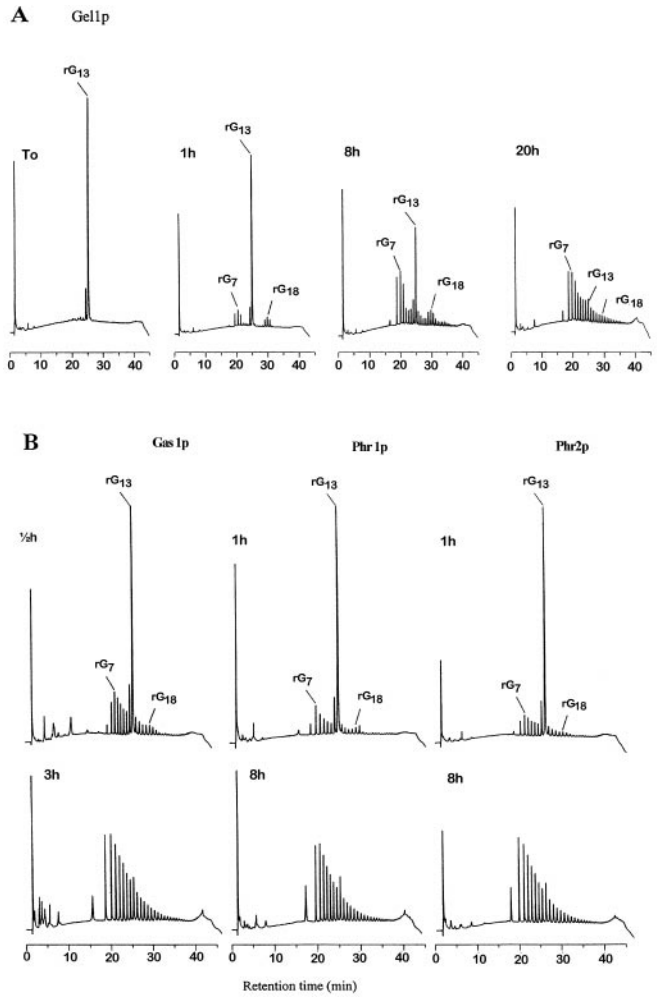

FIG. 4. **HPAEC analysis of products from the incubation of the recombinant Gel1p, Gas1p, Phr1p, and Phr2p with reduced laminarioligosaccharides.** 3, 3, 1, and 2 μg of respective purified recombinant proteins were incubated with 3 mM reduced laminarioligosaccharide containing 13 glucose units ($_rG_{13}$) 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 (T_0 identical to *A*).

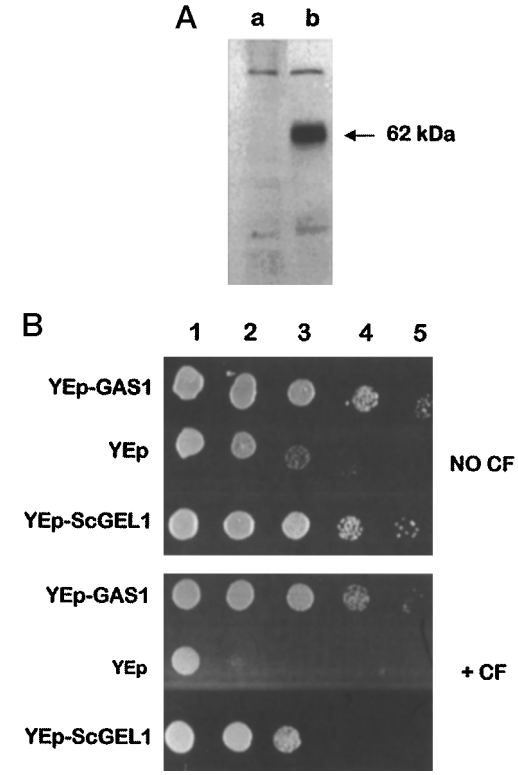

FIG. 5. *A,* immunoblot analysis of *S. cerevisiae* extracts. Total extracts from exponentially growing cells of WB2d (YEp) strain (*lane a*) and of WB2d (YEp-*ScGEL1*) (*lanes b*) were analyzed by immunoblotting with anti-Gel1p antibodies. The same amount of proteins was loaded on each lane. *B,* sensitivity to Calcofluor White. At a cell density of 5 × 10^6/ml, 5 μl of a concentrated suspension of cells (*lane 1*), and of 10× (*lane 2*), 100× (*lane 3*), 1,000× (*lane 4*), and 10,000× (*lane 5*) dilution was spotted on standard minimal plates and minimal medium plates supplemented (*CF*) or not (*no CF*) with 50 μg/ml Calcofluor White.

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. 4*B*). 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 laminarioligosaccharides of different size. The minimum size of the laminarioligosaccharide recognized by the enzyme-binding site varied slightly with the protein; it was 10, 9, 10, and 11 glucose units for Gas1p, Phr1p, Phr2p, and Gel1p, respectively (data not shown).

Gelp 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 Δ*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. 5*A*). The absence of this band in the Δ*gas1* 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. 2*B*). 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 Δ*gas1*; 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. 5*B*, *GEL1* almost completely abolished the Δ*gas1* hypersensitivity to growth in the presence of Calcofluor White. Complementation of the Δ*gas1* mutant by

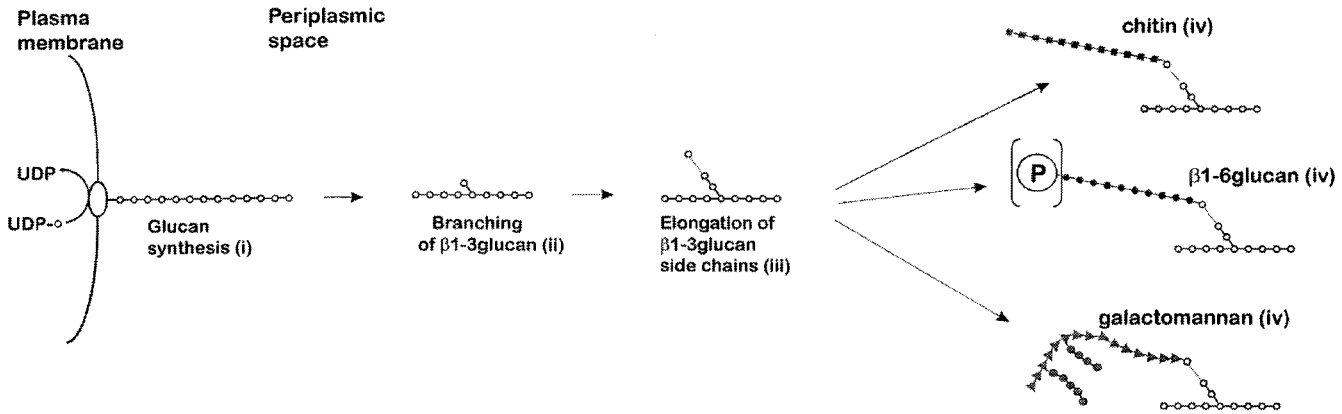

FIG. 6. **Chronological events involved in the synthesis and postsynthetic modifications of the cell wall 1,3-β-glucans.** (*i*) Biosynthesis of linear 1,3-β-glucans; (*ii*) branching of 1,3-β-glucans through 1,6-β linkages; (*iii*) elongation of 1,3-β-glucan side chains; and (*iv*) cross-linking of other polymers (chitin and galactomannan in *A. fumigatus*, or chitin, 1,6-β-glucan, and proteins in *S. cerevisiae*) onto non-reducing ends of 1,3-β-glucan side chains. *P*, proteins.

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 Δ*gas1* 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 Δ*gas1* 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), Δ*gas1* cells were almost completely resistant to the enzyme, as previously reported (9). The wild-type strain was very sensitive with a 85% decrease in $A_{660\ nm}$, 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 Δ*gas1* mutation in *S. cerevisiae* (Ref. 18 and this study). (ii) Engineered expression of *PHR1* in a Δ*phr2* mutant strain and *PHR2* in Δ*phr1* 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*, Δ*gas1* 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-glucosylated 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 Δ*phr1* mutant is not able to conduct apical growth of either yeast or hyphal growth forms. Cells become larger and rounder, a phenotype reminiscent of the Δ*gas1* 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 Δ*phr1* 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 Δ*gas1* mutant, Δ*phr1* mutant is hypersensitive to Calcofluor White and to Nikkomycin Z.

In contrast to *PHR1*, *PHR2* is only expressed at acidic pH (≤5) (12). A Δ*phr2* 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 Δ*phr2* mutant.

In *C. maltosa*, a Δ*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 Δ*gas1* mutant (9, 25). At pH 7, no morphological differences were noted. Transition of yeast to pseudohyphal growth was abolished in the Δ*epd1* mutant at pH 4 but not at pH 7. This pattern is somehow reminiscent of Δ*phr2* mutant except that Δ*epd1* mutant grows at pH 4, whereas Δ*phr2* does not. The cell wall of the Δ*epd1* mutant is characterized by an increase in chitin and a decrease in 1,6-β-glucans. However, in contrast to Δ*phr1* and Δ*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*[3] and previous studies on the cell wall of *S. cerevisiae* (37, 38) suggested that the chronological events involved in the synthesis and postsynthetic modifications of the cell wall 1,3-β-glucans are as follows: (i) biosynthesis of linear 1,3-β-glucans; (ii) branching of 1,3-β-glucans through 1,6-β linkages; (iii) elongation of 1,3-β-glucan side chains; and (iv) cross-linking of other polymers (chitin and galactomannan in *A. fumigatus* or chitin and 1,6-β-glucan and proteins in *S. cerevisiae*) onto the nonreducing ends of 1,3-β-glucan side chains (Fig. 6).

Our working hypothesis for the biological function of Gelp, Gasp, and Phrp is that the 1,3-β-glucanosyltransferase is involved in the elongation of 1,3-β-glucan side chains (step iii in the biosynthetic pathway). By doing so, it would increase the number of 1,3-β-glucan side chains with free non-reducing ends available for cross-linking with other polysaccharides. Based on this model, mutation in the encoding gene(s) in yeast would induce perturbations in the cross-linking events in the cell wall as follows: (i) the reduction of the 1,3-β-glucan and 1,6-β-glucan concentration in the alkali-insoluble fraction leading to an increase in the ratio of alkali-soluble to alkali-insoluble glucans; (ii) the secretion in the culture medium of β-glucan and glycoproteins normally covalently bound to the branched polysaccharide core of the cell wall. These events have been observed in Δ*gas* and Δ*phr* mutants. Increases of the chitin level and its cross-linking to 1,6-β-glycosylated mannoproteins also seen in these mutants are expected to be due to a set of compensatory reactions to palliate the cell wall defect due to the mutation. Such compensatory changes seem common in cell wall (33, 39).

Another putative function for *GEL/GAS/PHR* could be a role in cell expansion since null yeast mutants showed altered polarized growth. Hydrolysis of the existing cell wall structure is obviously required for hyphal branching, conidial germination, or yeast buddings and the way the fungal cell wall is plasticized remains to date a mystery. Indeed, all null 1,3-β-glucanase mutants obtained up to date do not have a phenotype (40).[4] The glucanosyltransferase activity could be responsible for such modifications in the cell wall and be responsible for an equilibrium between hydrolysis of cell wall polymers and elongation of existing polymers allowing for an apical growth.

This study emphasized the role of GPI-anchored proteins in cell wall morphogenesis. A dual function has been now established for these proteins: some members of this family (such as Gel1p, Gas1p, Phr1p, and Phr2p) have a 1,3-β-glucanosyltransferase activity responsible for the modification of the existing polymers of the cell wall, whereas others, such as Krep, are involved in the *de novo* synthesis of new polymers. Some GPI-anchored proteins without known enzymatic function (like Agα1, Cwp1p, or Cwp2p) are covalently 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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