The Essential Smp3 Protein is Required for Addition of the Side-Branching Fourth Mannose during Assembly of Yeast Glycosylphosphatidylinositols

Stephen J. Grimme, Barbara A. Westfall, Jill M. Wiedman, Christopher H. Taron, and Peter Orlean‡.

From the Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, U.S.A.

Running title: Essential Smp3p adds Fourth Mannose to Yeast GPI Precursors

‡ To whom correspondence should be addressed:
Department of Biochemistry
University of Illinois at Urbana-Champaign
309 Roger Adams Laboratory
600 South Mathews Avenue
Urbana, IL 61801

Tel: 217-333-4139
FAX: 217-244-5858
E-mail: p-orlean@uiuc.edu.
SUMMARY

The major glycosylphosphatidylinositols (GPIs) transferred to protein in mammals and trypanosomes contain three mannoses. In *Saccharomyces cerevisiae*, however, the GPI transferred to protein bears a fourth, α1,2-linked Man on the α1,2-Man that receives the phosphoethanolamine (EthN-P) moiety through which GPIs become linked to protein. We report that temperature-sensitive *smp3* mutants accumulate a GPI containing three mannoses and that *smp3* is epistatic to the *gpi11, gpi13*, and *gaa1* mutations, which normally result in the accumulation of Man$_4$-GPIs, including the presumed substrate for the yeast GPI transamidase. The Smp3 protein, which is encoded by an essential gene, is therefore required for addition of the fourth Man to yeast GPI precursors. The finding that *smp3* prevents the formation of the Man$_4$-GPI that accumulates when addition of EthN-P to Man-3 is blocked in a *gpi13* mutant suggests that the presence of the fourth Man is important for transfer of EthN-P to Man-3 of yeast GPIs. The Man$_3$-GPI that accumulates in *smp3* is a mixture of two dominant isoforms, one bearing a single EthN-P side branch on Man-1, the other with EthN-P on Man-2, and these isoforms can be placed in separate arms of a branched GPI assembly pathway. Smp3-related proteins are encoded in the genomes of *Schizosaccharomyces pombe*, *Candida albicans*, *Drosophila melanogaster*, and *Homo sapiens*, and form a subgroup of a family of proteins whose other groups are defined by the Pig-B(Gpi10) protein, which adds the third GPI mannose, and by the Alg9 and Alg12 proteins, which act in the dolichol pathway for N-glycosylation. Because Man$_4$-containing GPI precursors are normally formed in yeast and *Plasmodium falciparum*, whereas addition of a fourth Man during assembly of mammalian GPIs is rare and not required for GPI transfer to protein, Smp3p-dependent addition of a fourth Man represents a target for antifungal and antimalarial drugs.
INTRODUCTION

Glycosylphosphatidylinositol (GPI) with the conserved core structure \( \text{H}_2\text{N-CH}_2\text{-CH}_2\text{-PO}_4\text{-6Man}\alpha_1,2\text{Man}\alpha_1,6\text{Man}\alpha_1,4\text{GlcN}\alpha_1,6\text{Ins} \) phospholipid are made by all eukaryotes. Many GPIs are transferred to the COOH terminus of secretory glycoproteins and serve as membrane anchors, but others remain protein-free (1-3). In *Saccharomyces cerevisiae*, mannoproteins can be transferred from their GPI anchor to cell wall \( \beta \)-glucan (4,5). The formation of GPIs is essential for growth of yeasts, *Leishmania mexicana*, the bloodstream form of *Trypanosoma brucei*, and for embryonic development in mammals (6-10).

GPIs are preassembled on an inositol phospholipid in a stepwise pathway whose enzymes are localized in membranes of the endoplasmic reticulum (ER) (2, 3,11,12). During its assembly, the glycan core of the GPI precursor can be decorated with side branches. In yeast and mammals, the first and second mannoses can be modified with phosphoethanolamine (EthN-P) (11-23), and late stage GPI precursors made in wild type *Saccharomyces cerevisiae* and *Plasmodium falciparum* cells late-stage GPI precursors bear a fourth, \( \alpha 1,2 \)-linked Man on the third, \( \alpha 1,2 \)-linked Man of the glycan core (24-26). Yeast mutants deficient in EthN-P addition to Man-2 and Man-3 and in GPI transfer to protein accumulate GPIs with four mannoses (21, 22, 27, 28). In contrast, in mammalian cells, the largest glycan headgroups characterized contain only three mannoses (13-18), although it has been suggested that minor amounts of \( \text{Man}_4 \) species may be formed (13, 18, 29). Protein-bound GPIs in mammals usually contain three mannoses, but a fourth, \( \alpha 1,2 \)-linked Man has been detected on the GPI anchor of the murine Thy-1 glycoprotein (30) and human renal membrane dipeptidase (31).

We report that the *S. cerevisiae* Smp3 protein, a member of a family of potential dolichyl phosphate (Dol-P) Man-utilizing mannosyltransferases, is a candidate for the enzyme that adds the fourth Man during yeast GPI assembly. Temperature-sensitive *smp3* mutations prevent the formation of \( \text{Man}_4 \)-GPIs and result in the accumulation of \( \text{Man}_3 \)-GPIs. Addition of the fourth mannose is therefore required to generate the \( \text{Man}_4 \)-GPIs that
are the most likely substrates for the yeast GPI transamidase. Because the SMP3 gene is essential, and because addition of a fourth Man seems to be of much greater relative importance in GPI assembly in yeast than it is in mammalian cells, addition of the fourth Man represents a potential target for antifungal drugs.

**EXPERIMENTAL PROCEDURES**

**Materials** - [2-³H]myo-inositol (sp. act.: 15-20 Ci/mmol) and [1-³H]ethanolamine (sp. act.: 10-30 Ci/mmol) were obtained from American Radiolabeled Chemicals, St. Louis, MO. Tran³5S-labeling mixture (sp. act.: 1 Ci/mmol) and phosphatidylinositol-specific phospholipase C were from ICN Biochemicals and Jack Bean and Aspergillus satoi α-mannosidases from Oxford GlycoSciences, Oxford, U.K. Silica gel 60 HPTLC 5631/5 plates were supplied by Altech, Deerfield, IL.

**Yeast Strains and Media** - The yeast strains used in this work are listed in Table 1, and the construction of those strains made specifically for this study is detailed below. YPD and SD medium were prepared as described in ref. 32, and YPGal medium had the same composition as YPD, but contained 2% (w/v) galactose instead of glucose. SGlyYE, SGlcYE, and SGalYE were as described in ref. (22), except that SGlcYE contained 2% (w/v) glucose.

The smp3 strain KH8 (33) was back-crossed twice to strain YMW2 (34) to generate the smp3-1 and smp3-1D strains. The smp3-2 and gaa1-2 strains were isolated in a screen for mutants that require the GPI1 gene for growth², conducted using the vectors and strains described in ref. (34). Both primary isolates were back-crossed twice to strain YMW1. The cross of smp3-1 to ∆gpi1-8H yielded viable smp3-1/∆gpi1 haploids. An smp3/gpi11 strain was generated by crossing smp3-1 with strain ∆gpi11-pPIG-F (22).

smp3/gpi13 "double mutants" were created by crossing haploid smp3-1 or smp3-2 strains with a haploid gpi13::KAN³-pGAL-GPI13 strain (22) and allowing the resulting diploids to sporulate. Asci were dissected and ascospores allowed to germinate on YP-
galactose medium supplemented with 0.25 M KCl. The genotypes of segregants from tetratype tetrads were determined from the segregation of selectable markers and temperature-sensitivity among these strains, and confirmed by the thin-layer chromatographic profiles of their [3H]inositol-labeled lipids. The "double mutants" grew more slowly at 25°C on solid YPGal medium than the single mutants unless osmotic support was provided.

smp3/gaa1 double mutants were generated in a genetic cross of the smp3-1 and gaa1-2 strains. The smp3/gaa1 segregants from tetratype tetrads were distinguishable from the smp3-1 and gaa1-2 single mutation because, unlike the single mutants, they failed to grow at 37°C on solid YPD medium supplemented with 0.25M KCl. The presence of both the smp3 and gaa1 mutations in these strains was confirmed by introducing the SMP3 gene on a plasmid: at non-permissive temperature, the double mutants showed the profile of [3H]inositol-labeled lipids characteristic of gaa1, not smp3 (see Fig. 2A).

smp3-1 was crossed into an ethanolamine-auxotrophic Δpsd1/Δpsd2 strain background (RYY51; ref. 35) to generate a strain capable of enhanced incorporation of exogenous ethanolamine into lipid. Media for growth of the smp3-1/Δpsd1/Δpsd2 strain were supplemented with 2 mM ethanolamine and 2 mM choline.

A heterozygous smp3::KarR/SMP3 disruptant, generated by the Saccharomyces Deletion Project, was obtained from Research Genetics, Huntsville, AL. Plasmid pSMP3-426, in which SMP3 is expressed behind its native promoter, was made by using PCR to amplify a genomic DNA fragment containing SMP3 and 401 nucleotides immediately upstream of the start codon and 336 nucleotides immediately downstream of the SMP3 coding region, after which the DNA fragment was cloned into the 2μ plasmid pRS426 (36). Plasmid pGAL-SMP3, in which expression of SMP3 is under the control of the glucose-repressible GAL10 promoter, was made by using PCR to amplify the SMP3 coding region from genomic DNA, and cloning the resulting DNA fragment between the GAL10 promoter and the GAL7 termination region of plasmid pMW20 (34), yielding plasmid
pGAL-\textit{SMP3}. Haploid \textit{smp3::Kan} strains complemented by \textit{pSMP3-426} or \textit{pGAL-SMP3} were generated by introducing these plasmids into the \textit{smp3::Kan}/\textit{SMP3} strain, allowing the diploids to sporulate, and recovering kanamycin-resistant \textit{smp3::Kan}-\textit{pSMP3-426} or \textit{pGAL-SMP3} strains from the tetrads that yielded four viable segregants. Both plasmids therefore expressed functional Smp3p.

A \textit{gpi10::LEU2/GPI10} diploid was generated by replacing 95\% of the \textit{GPI10} coding region with the selectable marker \textit{LEU2} using the strategy previously used to disrupt the \textit{GPI11} gene (22). Plasmid \textit{pGPI10-426}, in which \textit{GPI10} is expressed behind its native promoter, was made by using PCR to amplify a genomic DNA fragment containing \textit{GPI10} and 465 nucleotides immediately upstream of the start codon and 337 nucleotides immediately downstream of the \textit{GPI10} coding region, after which the DNA fragment was cloned into the 2µ plasmid pRS426. This plasmid was introduced into \textit{gpi10::LEU2/GPI10} diploids, which were then allowed to sporulate. The resulting asci were dissected, and \textit{gpi10::LEU2-pGPI10-426} segregants were recovered from tetrads that gave four viable segregants, indicating that \textit{pGPI10-426} expressed functional Gpi10p.

\textbf{Radiolabeling of Lipids-} For [\textsuperscript{3}H]inositol-labeling, logarithmically growing cells were resuspended at 10 OD \textsubscript{600} units/ml in inositol-free synthetic medium, shifted as appropriate to non-permissive temperature for 20 min, then labeled with 15 \textmu Ci [\textsuperscript{3}H]inositol for 90 min. For [\textsuperscript{3}H]inositol-labeling of GPs in the \textit{smp3::Kan}-\textit{pGAL-SMP3} strain, cultures were grown in SDGlyYE medium, cells then resuspended in SDGlcYE medium for 16 h to repress \textit{SMP3} expression, and cultures then pulse-labeled with [\textsuperscript{3}H]inositol for 2 h as detailed in ref (22). In the case of the \textit{smp3/Δgpi13-pGAL-SMP3} strain, \textit{GPI13} expression was likewise repressed by incubation in SDGlcYE medium at 25°C, after which portions of the culture were either maintained at 25°C or shifted to 37°C for 20 min before pulse-labeling with [\textsuperscript{3}H]inositol (22). For [\textsuperscript{3}H]ethanolamine-labeling, strains were grown in SD medium supplemented with choline and ethanolamine, then washed and radiolabeled with 50 \textmu Ci ethanolamine in SD medium lacking ethanolamine. Radiolabeled
lipids were extracted and separated by TLC using chloroform/methanol/water (10:10:2.5 by volume) as solvent (22).

**Characterization of Glycan Headgroups** - [³H]inositol-labeled lipids from 500 OD₆₀₀ units of smp3-1 cells labeled at 25°C were purified by two rounds of preparative TLC. Size analyses of the neutral glycan headgroup of the de-acylated lipid, Jack Bean- or Aspergillus satoi α₁,₂ mannosidase-sensitivity determinations, and positioning of EthN-P side-branches were carried out following protocols described in refs. 22 and 37. A Man₄-GlcNAc standard was obtained from the Man₄-GPI that accumulates in the gpi7-deleted strain (21, 22), and a Man₃-GlcNAc standard was prepared isolated after Jack Bean α mannosidase treatment followed by dephosphorylation of the deacylated gpi7 lipid.

[³⁵S]-Labeling and Immunoprecipitation of Gas1p - Pulse-labeling with Tran³⁵S labeling mixture, chasing with unlabeled methionine and cysteine, and immunoprecipitation of Gas1p were carried out as described previously (38).

**Amino Acid Sequence Analyses** - The amino acid sequences of other eukaryotic proteins resembling *S. cerevisiae* Smp3p (NP_014792), Gpi10p (NP_011373), Alg9p (NP_014180), and Alg12p (NP_014427) were identified in Psi-BLAST, BLASTp, or tBLASTn searches (39, 40) of the NCBI, Sanger Centre, and Stanford DNA Sequencing and Technology Center databases. Each new amino acid sequence was used as probe in a Psi-BLAST search of all *S. cerevisiae* proteins, and the new protein was then provisionally designated the counterpart of the *S. cerevisiae* protein with which it gave the alignment with the lowest E value and to which it showed the highest level of amino acid identity and similarity. The accession numbers of the *S. pombe* Smp3p, Gpi10p, Alg9p, and Alg12p counterparts are Q09837, T41079, T50116, and T39659, respectively. The accession numbers of the *Drosophila* Smp3p, Gpi10p, Alg9p, and Alg12p counterparts are AAF47201, AAF47795, AAF56419, and AAF54441, respectively. The accession numbers of the human Smp3p, Gpi10p, Alg9p, and Alg12p counterparts are BAB14263, NP_004846 (Pig-B), BAB15154, and AAH01729 respectively. The *C. albicans*
proteins are encoded by the following contigs of genomic DNA from *C. albicans* strain SC5314, sequenced by the Stanford DNA Sequencing and Technology Center: CaSmp3p by nucleotides 5967-7460 of Contig6-2467, CaGpi10p by nucleotides 14358-15803 of contig6-2493, CaAlg9p by nucleotides 40345-42024 of Contig6-2488, and CaAlg12p by nucleotides 38706-40457 of Contig6-2478. Sequence data for *Candida albicans* were obtained from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/candida. Sequencing of *Candida albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. Amino acid sequences were aligned using the CLUSTAL W program (41), and an unrooted phylogenetic tree was generated from that alignment using the DRAWTREE option of the PHYLIP program (42), including positions with gaps, and not correcting for multiple substitutions or using branch lengths.

**RESULTS**

Identification of Smp3p as a Candidate GPI Mannosyltransferase—Because the fourth Man is added to the yeast GPI precursor in the ER (24), and because the three core mannoses added to the GPI precursor in the ER are donated by Dol-P-Man (2, 3, 11, 43), the fourth Man on yeast GPls might also be expected to originate from Dol-P-Man. Two lines of evidence suggested that the Smp3 protein might be a Dol-P-Man-utilizing mannosyltransferase involved in GPI assembly. First, the *S. cerevisiae* genome encodes several proteins similar to Pig-Bp (19, 20, 43, 44), which is required for addition of the α1,2-linked Man to mammalian GPls (43). Of these, Gpi10p is the functional homolog of Pig-Bp (19, 20), and Alg9p and Alg12p are non-essential proteins involved in mannosyl transfer to the dolichol-linked precursor in N-glycosylation (45). The *SMP3* gene encodes a fourth related protein. The temperature-sensitive *smp3-1* mutant had previously been isolated in a screen for yeast mutants that stably maintained a heterologous plasmid (33), and its availability allowed us to test it for a defect in GPI anchoring. Genetic evidence implicating
Smp3p in GPI anchoring came from the fact that we isolated an allele of \textit{SMP3} in a screen for mutants that require the \textit{GPI1} gene for growth\textsuperscript{2}. Gpi1p, which is necessary for growth at 37°C but not 25°C, is a subunit of the protein complex that catalyzes the first step in GPI assembly, the formation of GlcNAc-PI (38, 46). The premise behind our synthetic lethality screen was that an additional mutation affecting GPI assembly or transfer to protein might be lethal when combined with the \textit{\textDelta gpi1} mutation. One temperature-sensitive strain isolated in this way was complemented by a centromeric plasmid (47) containing \textit{SMP3} that we recovered from a genomic yeast DNA library. We confirmed by integrative transformation that the cloned \textit{SMP3} gene was closely linked to the locus conferring temperature-sensitivity, and we refer to this mutation as \textit{smp3-2}.

The temperature-sensitive growth phenotype of both the \textit{smp3-1} and \textit{smp3-2} alleles was osmotically remediable: inclusion of either 0.25 M KCl or 1 M sorbitol in solid medium restored the ability of these strains to grow at 37°C. Although \textit{SMP3} was shown to be an essential gene (33), we tested whether haploid \textit{smp3} disruptants might be capable of vegetative growth if given osmotic support. Heterozygous \textit{smp3::Kan}\textsuperscript{R}/\textit{SMP3} diploids were allowed to sporulate, and the resulting asci were dissected onto solid YPD medium, or onto YPD medium supplemented with 0.6 M KCl or 1.0 M sorbitol. When ascospores were dissected onto YPD medium, only the two kanamycin-sensitive, wild type segregants gave rise to colonies, whereas the \textit{smp3::Kan}\textsuperscript{R} segregants either failed to germinate or failed to divide. When dissected onto osmotically supported YPD medium, the \textit{smp3::Kan}\textsuperscript{R} segregants completed 4 to 8 rounds of cell division, but then ceased further growth. We conclude that Smp3p is essential for vegetative growth.

\textit{smp3 Mutants Accumulate a Candidate GPI Precursor}- To establish whether \textit{smp3} mutants are defective in GPI synthesis, we tested whether they accumulate an intermediate in the GPI synthetic pathway. Accumulation of a precursor provides a more sensitive indication of a GPI assembly defect in late-stage yeast GPI anchoring mutants than testing for defects in GPI transfer to protein can. For example, the \textit{\textit{gpi10-1}} mutant accumulates a
Man₂-containing GPI precursor, but incorporates normal amounts of [³H]inositol into proteins (19).

The *smp3-1* and *smp3-2* mutants, a wild type strain, and *smp3* mutants harboring a centromeric library plasmid containing the *SMP3* gene were pulse-labeled with [³H]inositol at 25 and 37°C, after which labeled lipids were extracted from the cells, and separated by TLC. Both strains accumulate a major [³H]inositol-labeled lipid (“3-1”), as well as a minor, less polar species (“3-2”), suggesting that they have a defect in GPI synthesis (Fig. 1A, lanes 3, 4, 7, and 8). Accumulation of lipid 3-1 is highest at 25°C in the *smp3-1* strain, whereas *smp3-2* accumulates somewhat more at 37°C. Lipid accumulation by both *smp3* strains is abolished when the *SMP3* gene is introduced into these strains on a centromeric plasmid (Fig. 1A, lanes 5, 6, 9, and 10), strongly suggesting that their GPI synthetic defect is due to a mutation in the *SMP3* gene.

To show that the lipid accumulation phenotype of the temperature-sensitive *smp3* mutants can be mimicked by depleting Smp3p in an *smp3*-disrupted strain, we isolated a haploid *smp3::Kan* strain that was complemented by a plasmid-borne copy of *SMP3* under the control of the glucose-repressible *GAL10* promoter. A culture of *smp3::Kan*-pGAL-*SMP3* cells was incubated in repressing medium for 16 - 48 h to allow them to become depleted of Smp3p, after which they were pulse-labeled with [³H]inositol. The glucose-repressed cells accumulated a lipid with the same chromatographic mobility as lipid 3-1 accumulated (Fig. 1B, lanes 1 and 2), indicating that lipid 3-1 indeed accumulates as a consequence of the loss of Smp3p function. Lipid 3-2 was not unambiguously resolved in this experiment. Further lipid-labeling experiments were done with temperature-sensitive *smp3* strains.

Lipid 3-1 is sensitive to mild base hydrolysis but resistant to hydrolysis by phosphatidylinositol-specific phospholipase C (Fig. 1C). The former property indicates the lipid has ester-linked fatty acyl chains, and the latter is consistent with the presence of an acyl chain esterified to the inositol: both are features of late-stage yeast GPI precursors (19,
Lipid 3-1 could also be radiolabeled with $[^3]$H]ethanolamine, consistent with it being a GPI with one or more EthN-P moieties (Fig. 1D, lane 2).

**Epistasis Relationships of smp3-**. To obtain genetic evidence that lipid 3-1 is made in the GPI assembly pathway, we tested whether its formation is abolished in a mutant defective in the first step in GPI synthesis, and whether, in turn, smp3 blocks the formation of late-stage and “complete” GPI precursors. Double mutants were constructed between smp3 and i) $\Delta gpi1$, which blocks GlcNAc-PI synthesis, the first step in GPI assembly (38), ii) gpi11 (22), which is defective in the yeast counterpart of human Pig-Fp (48) and accumulates two Man$_4$-GPIs, iii) $\Delta gpi13$-pGAL-GPI13, in which depletion of Gpi13p blocks addition of phosphoethanolamine to Man-3 and leads to accumulation of a Man$_4$-GPI bearing EthN-P on its first Man (22), and iv) gaa1, which is blocked in GPI transfer to protein and accumulates the “complete” GPI precursor CP2 (28).

The smp3-1/$\Delta gpi1$ mutant does not accumulate lipid 3-1 at either 25 or 37°C (Fig. 2A, lanes 1-4), indicating that 3-1 accumulation is dependent on formation of GlcNAc-PI, and placing the smp3 block downstream of $\Delta gpi1$ in the GPI assembly pathway. smp3, however, is epistatic to gpi11, gaa1, and gpi13. In the smp3-1/gpi11 strain, formation of Man$_4$-GPIs “11-1” (which bears two EthN-Ps, one of which is on Man-3) and “11-2” (which bears a single EthN-P on Man-2; Fig. 2A, lane 5) is almost completely prevented (Fig. 2A, lanes 6 and 7). In the smp3-1/gaa1 double mutant, accumulation of the “complete precursor” CP2, a Man$_4$-GPI with three EthN-Ps (19) (Fig. 2A, lane 8), is essentially abolished (Fig. 2A, lanes 9 and 10). We noted that the smp3/gaa1 double mutant had a more severe growth defect than strains harboring the smp3-1 or gaa1-2 mutations alone, because, in contrast to the single mutants, the smp3/gaa1 strain did not grow at 37°C on solid YPD medium supplemented with 0.25M KCl. Strikingly, the smp3-1 and smp3-2 mutations inhibited the formation of the Man-Man-Man-(EthN-P)Man-GPI (“lipid 13-1”) that accumulates in Gpi13p-depleted strains (22), and did so in a temperature sensitive manner. After shifting the “double mutants” to 37°C, formation of lipid 13-1 was reduced in
the \textit{smp3-1/\Delta gpi13-pGAL-GPI13} strain, and abolished in \textit{smp3-2/\Delta gpi13-pGAL-GPI13} (Fig. 2B, lanes 2, 3, 5, and 6), and lipid 3-1 accumulated as it does in \textit{smp3} single mutants. The \textit{smp3} mutation therefore blocks the formation of the \textit{Man}_4-GPIs that accumulate in strains defective in each of three essential proteins that act late in the GPI anchoring pathway.

\textit{smp3 is Blocked in Addition of a Fourth Man to GPI Precursors}- The finding that \textit{smp3} blocks the accumulation of \textit{Man}_4-GPIs is consistent with the notion that the mutation prevents the addition of the fourth, $\alpha$-1,2-linked mannose to GPIs and led to the prediction that lipid 3-1 is a \textit{Man}_3-GPI. We tested this by determining the structure of the glycan headgroup of lipid 3-1. $[^3]$Hinositol-labeled 3-1 was isolated by preparative TLC, and its neutral glycan headgroup obtained following deacylation, HF-dephosphorylation, and re-$N$-acetylation. Portions of this material were submitted to size analysis by HPTLC without, or after, digestion with $\alpha$-mannosidases. The full-size glycan from lipid 3-1 has a mobility corresponding to that of \textit{Man}_3-GlcNAc-Ins (Fig. 3A, lane 2). JB$\alpha$M treatment converts the 3-1 glycan to GlcNAc-Ins, whereas AS$\alpha$M converts it to \textit{Man}_2-GlcNAc-Ins (Fig. 3B, lanes 2-5). These results indicate that lipid 3-1’s glycan contains three $\alpha$-linked mannoses, the outermost of which is in $\alpha$-1,2 linkage. \textit{smp3} is therefore indeed defective in the addition of the fourth, $\alpha$-1,2-linked mannose to the GPI precursor.

The finding that lipid 3-1 could be radiolabeled with $[^3]$Hethanolamine indicated the presence of one or more EthN-Ps on this \textit{Man}_3-GPI. To determine the position of its EthN-P side-branch(es), the deacylated 3-1 headgroup was first treated with JB$\alpha$M, then dephosphorylated and re-$N$-acetylated. This succession of treatments yielded two major glycans, one migrating as \textit{Man}_2-GlcNAc-Ins, the other as Man-GlcNAc-Ins (Fig. 3B, lane 6), indicating that lipid 3-1 is a mixture of structural isoforms. The recovery of Man-GlcNAc-Ins indicates that the GPI it originated from must have borne an EthN-P moiety on its first mannose, and therefore that only one EthN-P was present on the original GPI. Because the GPI that gave rise to \textit{Man}_2-GlcNAc-Ins comigrates with the one that yielded Man-
GlcNAc-Ins, this component of lipid 3-1 must likewise bear only one EthN-P side-branch. We conclude that lipid 3-1 consists predominantly of a mixture of two Man$_3$-GPI isoforms, one bearing EthN-P on Man-2, and one bearing EthN-P on its first Man. Because traces of material with the mobility of Man$_3$-GlcNAc-Ins are also present in the sample in Fig. 3B, lane 6, it is possible that the lipid 3-1 mixture also contains a small amount of a Man$_3$-GPI with a single EthN-P moiety on Man-3, although the presence of this material could also be explained by incomplete JB$_{aM}$ digestion.

smp3 Strains Have a Partial Defect in GPI Anchoring of Protein- We tested whether the smp3 mutants are defective in GPI attachment to protein by examining GPI anchor-dependent processing of Gas1p, a standard procedure for detecting a GPI anchoring defect in yeast (49). A block in GPI attachment to Gas1p in turn prevents maturation of a 125 kDa form of the protein in the Golgi, and causes Gas1p to remain in a core-glycosylated, 105 kDa form (49). The two forms of Gas1p are detected by pulse-labeling smp3 cells with [$^{35}$S]methionine at 25 or 37°C, then performing a chase during which cultures are maintained at 25 or 37°C, after which [$^{35}$S]-labeled Gas1p is immunoprecipitated. In the wild type control strain, only the 125kDa form of Gas1p was seen after the chase period (Fig. 4, lanes 1 and 2), whereas in the $\Delta$gpi1 control, only the 105 kDa protein was present after a 60 min chase at 37°C (Fig. 4, lane 3). The smp3-2 mutant showed a partial defect in Gas1p processing at 25°C (Fig. 4, lanes 4-6), as did the $\Delta$gpi1 mutant at 25°C (38). Gas1p maturation remained incomplete at 37°C in smp3-2 (Fig. 4, lanes 7-9). The partial block in Gas1p processing suggests that Smp3p is required for efficient transfer of GPls to protein.

Neither smp3-1, nor smp3-2, nor the $\Delta$smp3-pGAL-SMP3 strain shifted to glucose showed a discernable defect in [$^3$H]inositol incorporation into protein. The incompleteness of the Gas1p processing block and the lack of effect on [$^3$H]inositol incorporation into protein may reflect a degree of leakiness of the smp3 mutation or incomplete repression of SMP3 expression. Alternatively, a Man$_3$-GPI may be transferred to protein (see Discussion),
although if this is the case, these aberrant protein-bound GPIs do not allow the cells to grow under non-permissive conditions.

Smp3p-Related Proteins Form a Sub-Group of a Family of Candidate Dol-P-Man-Dependent Mannosyltransferases— Searches of sequence databases revealed that the genomes of *Schizosaccharomyces pombe*, *Candida albicans*, *Drosophila melanogaster* and *Homo sapiens* encode proteins resembling *S. cerevisiae* Smp3p. The accession numbers of these protein sequences are given in Experimental Procedures. The new Smp3ps show amino acid sequence similarity to the Alg9, Alg12, and Pig-B(Gpi10) proteins, but are unlikely to be the sequence and functional homologs of these putative mannosyltransferases because the *S. pombe*, *D. melanogaster*, and *C. albicans* genomes all encode obvious counterparts of the latter proteins.

To show that the Smp3p-related proteins are indeed a separate sub-group of the Alg9p/Alg12p/Pig-Bp(Gpi10p) family, we generated a CLUSTAL W alignment of the amino acid sequences of 20 of these proteins and used it to produce an unrooted phylogenetic tree using the DRAWTREE option of the PHYLIP program (42). This analysis confirmed that the Smp3p-related proteins represent a separate group, as do the proteins designated the counterparts of Alg9p, Alg12p, and Pig-Bp(Gpi10p) (not shown). The Smp3 proteins are also distinguishable from other members of the family of putative mannosyltransferases because they have a variation on an amino acid sequence motif (19) that characterizes these proteins. Thus, the sequence H\textsuperscript{313}QExRF in the five Smp3-related proteins is HKExRF in all Alg9, Alg12, and Pig-B(Gpi10) proteins. (The first amino acid in these blocks is numbered according to its position in *S. cerevisiae* Smp3p, and x denotes a less conserved amino acid.) The family of candidate mannosyltransferases seems to include only four easily recognizable relatives: our searches of eukaryotic protein sequences using various Smp3p, Alg9p, Alg12p, and Pig-Bp(Gpi10p) sequences as probes have so far detected only proteins with obvious amino acid sequence similarity to one or other of these four proteins.
We have not established whether the human Smp3p counterpart is the functional equivalent of yeast Smp3p, but note that the presence of a human gene for such a protein is consistent with the fact that traces of GPs that have been speculated to bear a fourth Man have been detected in lipid extracts of mammalian cells (13, 29).

_Smp3p and Gpi10p Do Not Substitute for One Another In Vivo_- If the Gpi10 and Smp3 proteins are mannosyltransferases, then they transfer mannose to closely-related GPI structures to form α-1,2 mannosidic linkages. We therefore tested whether these enzymes might exhibit any cross-specificity for each other’s acceptor if expressed at high levels in stains deficient in the other protein. However, overexpression of _GPI10_ behind its native promoter on a 2µ plasmid neither restored the ability of _smp3-1_ strains to grow at 37°C, nor had a discernable effect on the lipid accumulation phenotype of _smp3-1_. Further, _smp3_ null mutants could not be rescued by overexpression of _GPI10_, and conversely, _SMP3_ did not restore viability to _gpi10_ disruptants when present on a 2µ vector. Smp3p and Gpi10p therefore cannot substitute for one another _in vivo_ under the conditions used, indicating that these putative mannosyltransferases have a high degree of specificity for their respective acceptor GPs _in vivo_.

**DISCUSSION**

The key findings reported here are that the essential Smp3 protein is required for addition of the fourth Man to yeast GPI precursors, that in the absence of Smp3p function, transfer of EthN-P to the third GPI Man is severely, if not completely blocked, and that Smp3p function is needed for efficient transfer of GPI precursors to yeast protein.

_Function and Essential Role of Smp3p_- The simplest explanation for the GPI assembly defect in _smp3_ mutants and for the fact that Smp3p resembles other proteins implicated in the transfer of Man from Dol-P-Man to manno-oligosaccharides is that Smp3p is the mannosyltransferase that adds the fourth, α-1,2-linked Man during assembly of yeast GPs. Assuming that the primary biochemical defect in conditional or null _smp3_ mutants is in
the addition of the fourth Man to GPIs, then this mannosyl residue itself must be critical, either for completion of the substrate(s) of the transamidase complex that adds GPIs to protein, or, if not for formation of transfer-competent GPIs per se, then for some subsequent function of the protein-bound GPI. We discuss these possibilities below.

The evidence to date strongly indicates that the GPI(s) that are normally transferred to protein in yeast all bear a fourth, α-1,2-linked mannose residue. Thus, protein-bound GPIs in yeast bear at least four mannoses (50), and the gaa1 and gpi8 mutants, which are defective in GPI transfer to protein, accumulate Man₄-GPIs, the most prominent of which is the “complete precursor” Man-(EthN-P)Man-(EthN-P)Man-(EthN-P)Man-GlcN-(acyl-Ins)PI (27, 28). Because smp3 is epistatic to gaa1, as well as to gpi11 and gpi13, which both accumulate Man₄-GPIs, Smp3p may be required for the formation of all GPI transamidase substrates, or at least enough “Man₄-complete precursors” to maintain viability. The latter possibility explains the fact that the Smp3p-deficient strains we have tested show a partial defect in GPI transfer to protein. Despite the strong lipid accumulation phenotype in these strains, they might nonetheless be leaky and still capable of making Man₄ transamidase substrates and attaching them to protein, although at levels too low to support cell growth under non-permissive conditions. Another possibility, namely that the apparent leakiness of the smp3 deficient strains is due to the transfer of Man₃GPIs to protein, is considered below.

If the presence of the fourth Man is indeed obligatory for a GPI to serve as a transamidase substrate, then our results suggest a specific requirement for this residue, namely, that the fourth mannose is important or necessary for transfer of the “bridging EthN-P” to Man-3 of the GPI precursor. Thus, Gpi13p-deficient mutants, which are blocked in the addition of EthN-P to Man-3, accumulate a GPI with the structure Man-Man-Man-(EthN-P)Man-GlcN-(acyl-Ins)PI (lipid 13-1 in ref. 22; Fig. 2B, lane 1), suggesting that the acceptor GPI recognized by Gpi13p is this Man₄GPI. Our finding that the smp3-2 mutation abolishes the formation of any detectable lipid 13-1 in the smp3-2Δgpi13-pGAL-GPI13
strain (Fig. 2B, lane 6) in turn raises the possibility that Gpi13p requires its acceptor GPI to bear a fourth mannose. We note that if Gpi13p could act on a Man₃-GPI before Smp3p does, then a single smp3 mutant might be expected to show an accumulation of trimannosyl GPIs bearing two or more EthN-Ps, which is not observed.

Although the earliest consequence of an Smp3p deficiency is a severe impairment in the formation of Man₄-GPIs, including the presumed acceptor for addition of the “bridging EthN-P”, it is possible that the fourth Man plays its critical role after GPI transfer to protein. Thus, EthN-P may be added to Man-3 of Man₃-GPIs in smp3 mutants, and such Man₃-GPIs may, in turn, be transferred to protein. This would also explain the apparent leakiness of smp3 mutants with respect to GPI transfer to protein. Lipid 3-1 contains traces of material that could have originated from (EthN-P)Man-Man-Man-GlcN-(acyl-Ins)PI (Fig. 3B, lane 6), a species that could itself serve as GPI transamidase substrate and which would accumulate in the smp3/gaa1 double mutant. However, if (EthN-P)Man-Man-Man-GlcN-(acyl-Ins)PI were to receive additional EthN-P side-branches on Man-1 or Man-2 or both, and be converted to the Man₃-counterpart of “complete precursors”, then such species should have been detectable in significant amounts in some of our lipid radiolabeling experiments as polar GPIs with chromatographic mobilities distinct from those of the known yeast Man₄-GPIs. Traces of polar lipids that are potential aberrant GPIs are detectable in extracts of certain smp3 strains (Fig. 2B, lanes 5 and 6, asterisk), or become visible after prolonged exposure of TLC plates to X-ray film, but the amounts of these species are too small to verify whether they are Man₃-GPIs. Importantly, however, if such trace lipids are indeed Man₃-GPIs capable of being transferred to protein, they would be predicted to be present at much higher levels in the smp3/gaa1 double mutant: however, no new polar lipids accumulated when this transamidase-defective strain was shifted to non-permissive temperature (Fig. 2A, lane 10). We note too that the smp3/gaa1 double mutant has a more severe growth defect than either smp3 or gaa1 alone, suggesting that
Smp3p-dependent addition of the fourth Man is necessary for the generation of optimal GPI transamidase substrates.

Although our results provide little support for the notion that Man$_3$-GPIs bearing two or more EthN-Ps are formed, they do not exclude the possibility that (EthN-P)Man-Man-Man-GlcN-(acyl-Ins)PI can be transferred to protein in smp3 mutants. However, even if this occurred, and some or all of the GPI anchors on yeast protein were to contain only three mannoses, this would not be sufficient for growth, as indicated by the inviability of smp3 disruptants and the conditional lethality of the smp3-1 and smp3-2 strains. The inability of protein-bound Man$_3$-GPIs to support growth in turn would imply that the fourth mannose, which is normally present on all yeast GPI anchors (50), fulfills another, essential function after anchor transfer to protein. One possibility is that this additional Man is necessary for incorporation of certain mannoproteins into the yeast cell wall upon creation of a cross-link between a Man in the GPI and β-1,6 glucan (4, 5). The fourth Man, however, may not form the attachment point for all mannoproteins, because in some cell wall proteins, the linkage to β-glucan may be through the reducing end of a core GPI Man (5). Another possible role for the fourth mannose on protein-bound GPs, consistent with the partial Gas1p processing defect of smp3-2, is that the fourth mannose may be required for efficient transport of GPI anchored proteins from the endoplasmic reticulum.

Although we cannot yet pinpoint the essential role of Smp3p, the simplest reason why this protein is necessary is the one implied by the earliest discernable biochemical consequence of a block in the addition of the fourth Man to yeast GPI precursors, namely, an inability to form Man-Man-Man-(EthN-P)Man-GlcN-(acyl-Ins)PI, the presumed acceptor for EthN-P transfer to Man-3. This in turn implies that addition of the fourth Man is critical for addition of the “bridging EthN-P” to Man-3 in yeast. The incomplete block in GPI transfer to protein in smp3 mutants must then be attributed to the leakiness of these strains and their consequent ability to make and transfer some Man$_4$-“complete precursor” to protein, even though these mutants accumulate Man$_3$-GPIs lacking the “bridging EthN-P”. The gpi10-1
strain provides a precedent for this possibility: it has a strong lipid accumulation phenotype, yet exhibits neither an apparent GPI anchoring defect nor temperature-sensitivity for growth (19). Nonetheless, Gpi10p is an essential protein because it is required for addition of the third mannose during GPI precursor assembly (19, 20).

Implications of the Structural Heterogeneity of Lipid 3-1 for GPI Assembly in Yeast

The major GPIs that can be radiolabeled in yeast mutants can be arranged in a scheme that implies a branched assembly pathway (22), and each of the two major isoforms of lipid 3-1 can be placed in one of the branches (Fig. 5). The Man-Man-(EthN-P)Man-GlcN-(acyl-Ins)PI isoform of lipid 3-1 (lipid 3-1-1) can be inserted between the Gpi10p and Gpi13p steps in an “(EthN-P)Man-1” arm that is defined by a succession of intermediates from Man-(EthN-P)Man-GlcN-(acyl-Ins)PI (19, 20) to “complete precursor” that all bear EthN-P on Man-1 (19-23). The Man-(EthN-P)Man-Man-GlcN-(acyl-Ins)PI isoform of 3-1 (lipid 3-1-2) is a potential precursor of the Man$_4$-GPI with EthN-P on Man-2 that accumulates in the gpi11 mutant (22). A straightforward explanation for the formation of the latter two GPIs is that they are intermediates in an “(EthN-P)Man-2” arm of a branched pathway. However, no obvious precursor to lipid 3-1-2 has been identified in strains deficient in addition of the third GPI mannose. Thus, although the gpi10 mutant accumulates Man-(EthN-P)Man-GlcN-(acyl-Ins)PI, the likely precursor of lipid 3-1-1 (19, 20), strains with a Gpi10p-deficiency alone have not been reported to accumulate Man-Man-GlcN-(acyl-Ins)PI or (EthN-P)Man-Man-GlcN-(acyl-Ins)PI species that could serve as precursors of lipid 3-1-2. Although an as yet undiscovered Man-2-substituted GPI may be generated earlier, it is also possible that pathway branching occurs after Gpi10p-dependent addition of Man-3. This could involve an “isomerization” in which EthN-P is removed from Man-1 and one is added to Man-2, or the addition of EthN-P to Man-2 of an unsubstituted Man$_3$-GPI. Although the latter GPI has not been demonstrated unambiguously, it is possible that lipid 3-2 is indeed Man-Man-Man-GlcN-(acyl-Ins)PI (Fig. 5), but we have so far been unable to resolve sufficient amounts of lipid 3-2 for characterization of its glycan headgroup.
We cannot rule out the formal possibility that the Man-2-substituted GPIs of the “(EthN-P)Man-2” pathway are non-physiological lipids generated only in GPI assembly mutants. However, the formation of Man$_3$- and Man$_4$-GPIs lacking EthN-P on Man-1 suggests that addition of EthN-P to Man-1 is not absolutely required for transfer of the third, $\alpha$1,2-linked mannose, although treatment of cells with an apparent inhibitor of EthN-P addition to Man-1 leads to accumulation of Man-Man-GlcN-(acyl-Ins)PI (20).

**Smp3p and “Bridging EthN-P” Addition as a Targets for Antimicrobial Agents**

Because GPI synthesis is essential in fungi and protozoa (6-9), this process could be targeted by drugs that exploit differences between mammals and microbes in the enzymology of GPI assembly. One proposed target is the addition of the first Man in trypanosomal GPI biosynthesis, which, unlike mammals, does not require prior inositol acylation of GlcN-PI (51,52). The identification of a species-specific inhibitor of EthN-P transfer to Man-1 (20, 53) indicates that even the same step in GPI assembly can be inhibited selectively in different organisms, a finding that validates the search for further selective inhibitors.

Our results suggest that both Smp3p-dependent addition of the fourth mannose to GPI precursors and Gpi13p-dependent transfer of the “bridging EthN-P” to a Man$_4$-GPI are reactions that could be targeted selectively by antifungal agents. Thus, from the structures and relative abundance of the GPI precursors that can be detected in mammalian cells, it is clear that Man$_4$-GPI precursors are at best rarely formed, and that the “bridging EthN-P” is readily added to Man-3 of a Man$_3$-GPI (13-18, 29). Moreover, cell lines deficient in the mammalian counterparts of the GPI transamidase components Gpi8p and Gaa1p accumulate Man$_3$-GPIs with one to three EthN-P substituents, suggesting that the major mammalian GPI transamidase substrate has three mannoses (54, 55). This is consistent with the fact that many protein-bound GPIs in mammalian cells have only three mannoses (1, 51). The opposite holds in *S. cerevisiae*: all protein-bound GPIs bear a fourth, $\alpha$-1,2-linked mannose (50), the presumed GPI transamidase subunits are Man$_4$-
GPIs, and the results of the present study indicate that at best, only very small amounts of Man$_3$-GPIs bearing the “bridging EthN-P” can be formed. The apparent rarity of free Man$_4$-GPIs in mammalian cells raises the possibility that addition of a fourth mannose to GPI precursors may be dispensible, in contrast to yeast, whereas Smp3p-dependent addition of Man-4 is essential for viability. Smp3p therefore represents a potential target for antifungal agents, and indeed, a likely Smp3p counterpart with 35% identity and 54% similarity to *S. cerevisiae* Smp3p, is encoded in the genome of the fungal pathogen *Candida albicans*. We note that the GPI biosynthetic intermediates made by *Plasmodium falciparum* also include structures bearing a fourth mannose (25, 26): the transferase that adds this mannose may therefore also be a potential target for antimalarial agents.

The transferases that add the “bridging EthN-P” to fungal GPIs are also potential drug targets. The Gpi13 and Pig-O proteins, which are required for addition of EthN-P to Man-3 of yeast and mammalian GPI precursors respectively (22, 23, 29), appear to differ in their specificities for GPI glycans. Thus, Pig-Op transfers EthN-P to a Man$_3$-GPI, whereas we have presented evidence here that its sequence homolog, Gpi13p, shows strong, possibly absolute specificity for a Man$_4$-GPI as acceptor for the “bridging EthN-P”. This difference in acceptor specificity could, in principle, also be exploited in the development of antifungal drugs.

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REFERENCES


FOOTNOTES

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; Dol-P-Man, dolichol phosphate mannose; ER, endoplasmic reticulum; EthN-P, phosphoethanolamine; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; PI-PLC, phosphatidylinositol-specific phospholipase C; JBαM, Jack Bean α-mannosidase; ASαM, Aspergillus satoi α1,2-mannosidase.

2 B. A. Westfall and P. Orlean, unpublished.
FIGURE LEGENDS

FIG 1. smp3 mutants accumulate a candidate GPI precursor. A. Accumulation of [³H]inositol-labeled lipids and correction of this phenotype by SMP3. Cells were labeled with [³H]inositol at 25 or 37°C, and radiolabeled lipids were extracted, separated by TLC, and detected by fluorography. Lanes 1 and 2 contain lipids from wild type cells; lane pairs 3-4 and 7-8, lipids from smp3-1 and smp3-2 cells respectively; lane pairs 5-6 and 9-10, lipids from smp3-1 and smp3-2 cells harboring a centromeric plasmid expressing SMP3 (pSMP3) behind its native promoter. Odd-numbered lanes contain lipids labeled at 25°C, even numbered lanes display lipids radiolabeled at 37°C. B. Depletion of Smp3p leads to accumulation of lipid 3-1. The Δsmp3-pGAL-SMP3 strain was shifted to SGlcYE medium to repress SMP3 expression as described in Experimental Procedures, after which cultures were labeled with [³H]inositol. A control culture incubated in SGalYE was incubated and radiolabeled in parallel. Radiolabeled lipids were extracted, separated by TLC, and detected by fluorography. Lane 1: culture shifted to glucose, lane 2 culture shifted to galactose. C. Mild base-sensitivity and PI-PLC-resistance of lipid 3-1. Samples of lipids from smp3-1 cells labeled with [³H]inositol at 25°C were submitted to mild base hydrolysis in methanolic NH₃ (lane 2) or mock-treated with aqueous methanol (lane 1), or incubated with PI-PLC (lane 4) or mock-treated (lane 3). Remaining lipids were extracted and separated by TLC. D. Radiolabeling of lipid 3-1 with [³H]ethanolamine. The Δpsd1/Δpsd2 (WT, lane 1) and smp3-1/Δpsd1/Δpsd2 (s3, lane 2) strains were radiolabeled with [³H]ethanolamine, and the smp3-1/Δpsd1/Δpsd2 strain was labeled in parallel with [³H]inositol (s3, lane 3). Radiolabeled lipids were separated on the same TLC plate. o- indicates the origin of the chromatogram.

FIG 2. Epistasis relationships of smp3. A. The smp3-1, Δgpi1, gpi11, and gaa1 mutants (lanes 1, 2, 5, and 8, respectively), and smp3-1/Δgpi1 (lanes 3 and 4), smp3-1/gpi11 (lanes 6 and 7), and smp3-1/gaa1 (lanes 9 and 10) double mutants were labeled
with [3H]inositol at 25°C or at 37°C after a 20 min shift of the culture to 37°C, and radiolabeled lipids were extracted, separated by TLC, and detected by fluorography. Positions of lipids 3-1, 11-1, 11-2, and CP2 are indicated, and their structures described in the text. B. smp3/Δgpi13-pGAL-GPI13 strains were incubated for 16 h at 25°C in SGlcYE medium, and each culture then divided into two portions, one of which was maintained at 25°C, whereas the other was shifted to 37°C for 20 min before pulse-labeling with [3H]inositol. Radiolabeled lipids were then extracted and separated by TLC. Lanes 2 and 3: [3H]inositol-labeled lipids that accumulate in the smp3-1/Δgpi13-pGAL-GPI13 strain at 25 and 37°C, respectively. Lanes 5 and 6: lipids radiolabeled in the smp3-2/Δgpi13-pGAL-GPI13 strain at 25 and 37°C, respectively. Lipids radiolabeled in the Δgpi13-pGAL-GPI13 strain after shift to glucose-containing medium are displayed in lane 1, and lipids labeled in the gaa1 and smp3-2 mutants at 37°C are separated in lanes 4 and 7, respectively. The asterisk indicates the position of a trace, aberrant lipid in lanes 5 and 6.

**FIG. 3.** Characterization of the glycan headgroup of lipid 3-1. Strain smp3-1 was radiolabeled with [3H]inositol at 25°C, and [3H]inositol-labeled lipid 3-1 was isolated by preparative TLC and deacylated with mild base. A. Size analysis of the neutral glycan. The headgroup was re-N-acetylated with acetic anhydride, then dephosphorylated with 50% aqueous HF, and the glycan submitted to HPTLC (lane 2). Lanes 3 and 4 contain Man₄-GlcNAc-[3H]Ins (M4) and Man₃-GlcNAc-[3H]Ins (M3) size standards prepared from the Man₄ GPIs that accumulate in the Δgpi7 mutant (22). Lane 1 displays a series of NaB[3H]₄-reduced Glc₂ (G2)-Glc₆ (G6) oligomers. B. α-mannosidase-sensitivity and positioning EthN-P side branches. HF-dephosphorylated glycans were incubated JBαM (HF/JBαM, lane 3) or with Aspergillus satoi α1,2-mannosidase (HF/α1,2M, lane 5). Mock-incubated controls for the digestions are in lanes 2 and 4. A sample of deacylated, re-N-acetylated headgroup was first treated with JBαM, then with HF (JBαM/HF, lane 6). Glycans were separated by HPTLC and detected by fluorography. M0, Ins, M1, M2, M3,
and M4 indicate the positions of GlcNAc-Ins, inositol, Man-GlcNAc-Ins, Man$_2$-GlcNAc-Ins, Man$_3$-GlcNAc-Ins, and Man$_4$-GlcNAc-Ins respectively. The mobilities of the GPI glycans relative to the [$^3$H]Glc oligomers correspond to those previously published (27).

FIG. 4. **smp3 is partially defective in Gas1p processing.** smp3-2 and wild type cells were pulse-labeled at 25 or 37°C, and Δgpi1 cells were labeled at 37°C with Tran[$^{35}$S]labeling mixture. Samples of the smp3 cultures were taken at the end of the pulse-labeling period (time = 0, lanes 4 and 7). The radioactivity in the labeled cultures was then chased with unlabeled amino acids for 30 or 60 min at 25 (lanes 5 and 6) or at 37°C (lanes 8 and 9). [$^{35}$S]-labeled Gas1p was then immunoprecipitated from extracts of the cells, separated by SDS-polyacrylamide gel electrophoresis, and detected by fluorography. Lanes 1 and 2: [$^{35}$S]Gas1p immunoprecipitated from the wild type control strain after a 60 min chase. Lane 3: [$^{35}$S]Gas1p from Δgpi1 cells after a 60 min chase.

FIG. 5. **Model for assembly pathway for yeast GPI assembly and site of the smp3 block.** Illustrated are the GPI structures that have been determined in detail (19-23) and the steps blocked in GPI anchoring mutants. Dashed arrows indicate the routes of the two putative pathway branches: it is not known whether both pathways lead to protein-bound GPls, or whether some GPls remain “free”. The precursor of lipid 3-1-2 is unknown, but possible intermediates in its synthesis are italicized, and the possibility that lipids 3-1-1 and 3-1-2 can be interconverted is indicated by a double-headed arrow. The structures of the GPl(s) that accumulate in mcd4 have not been determined, but mcd4 is likely to be defective in EthN-P addition to Man-1 (18, 20, 56). The abbreviations are: M, mannose, G; glucosamine; P, phosphate; E, ethanolamine.

**TABLE 1.** *Saccharomyces cerevisiae* strains used in this study. The procedures used to construct new strains for this study and references to previous descriptions of strains are given in Experimental Procedures.
Figure 1A

A

1  2  3  4  5  6  7  8  9  10

3-2

3-1

0-

25  37  25  37  25  37  25  37  25  37°C

WT  smp3-1  smp3-1  smp3-1  smp3-2  smp3-2  smp3-2  + pSMP3  + pSMP3
Figure 1 B, C, D

B

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C

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Figure 3

A

Treatment: HF

G2 G3 G4 G5 G6

B

HF HF HF HF HF JBαM

α1,2M HF

M0 Ins M1 M2 M3 M4
Figure 5
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<td>smp3-2 -pSMP3</td>
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<td>$\text{smp3-2},\text{MAT},\alpha,\text{ade2-1},\text{ade3},\Delta22,\text{his3-11,15},\text{leu2-3,112},\text{trp1-1},\text{ura3-1},\text{can},1-100$</td>
</tr>
<tr>
<td>smp3::KAN $^R$ /SMP3</td>
<td>-</td>
<td>$\text{smp3::KAN},\alpha,\text{SMP3},\text{MAT},\alpha,\Delta,\text{his3},\Delta,\text{leu2},\Delta,\text{met15},\Delta,\text{ura3},\Delta,\text{lys2},\Delta,0$</td>
</tr>
<tr>
<td>smp3::KAN $^R$</td>
<td>pGAL-SMP3</td>
<td>$\text{smp3::KAN},\alpha,\text{SMP3},\text{MAT},\alpha,\Delta,\text{his3},\Delta,\text{leu2},\Delta,\text{met15},\Delta,\text{ura3},\Delta,\text{lys2},\Delta,0$</td>
</tr>
<tr>
<td>gpi10::LEU2/GPI10</td>
<td>-</td>
<td>$\text{gpi10::LEU2/GPI10},\text{MAT},\alpha,\alpha,\text{ade2-1},\text{ade3},\Delta22,\text{his3-11,15},\text{leu2-3,112},\text{trp1-1},\text{ura3-1},\text{can},1-100$</td>
</tr>
<tr>
<td>RYY51</td>
<td>-</td>
<td>$\text{psd1::TRP1},\text{psd2::HIS3},\text{MAT},\alpha,\text{his3},\text{leu2},\text{lys2},\text{trp1},\text{suc2},\Delta9,\text{ura3}$</td>
</tr>
<tr>
<td>smp3-1 /Δpsd1 /2</td>
<td>-</td>
<td>$\text{smp3-1},\text{psd1::TRP1},\text{psd2::HIS3},\text{MAT},\alpha,\text{ade2-1},\text{his3},\text{leu2},\text{trp1},\text{lys2},\text{ura3}$</td>
</tr>
<tr>
<td>Δgpi1- 8H</td>
<td>-</td>
<td>$\text{gpi1-},\text{MAT},\alpha,\text{ade2-1},\Delta22,\text{ura3-1},\text{his3-11,15},\text{trp1-1},\text{leu2-3,112},\text{can},1-100$</td>
</tr>
<tr>
<td>Δgpi11 -pPIG-F</td>
<td>pPIG-F</td>
<td>$\text{gpi11::LEU2},\text{MAT},\alpha,\text{ade2-1},\text{ade3},\Delta22,\text{ura3-1},\text{his3-11,15},\text{trp1-1},\text{leu2-3,112},\text{can},1-100$</td>
</tr>
<tr>
<td>gaa1-2 -2C</td>
<td>-</td>
<td>$\text{gaa1-2},\text{MAT},\alpha,\text{ade2-1},\text{ade3},\Delta22,\text{ura3-1},\text{his3-11,15},\text{trp1-1},\text{can},1-100$</td>
</tr>
<tr>
<td>smp3-1 /Δ gpi1</td>
<td>-</td>
<td>$\text{smp3-1},\text{gpi1::TRP1},\text{MAT},\alpha,\text{ade2},\text{ade3},\Delta22,\text{his3-11,15},\text{leu2-3,112},\text{trp1-1},\text{ura3-1},\text{can},1-100$</td>
</tr>
<tr>
<td>smp3-1 /gpi11</td>
<td>pPIG-F</td>
<td>$\text{smp3-1},\text{gpi11::LEU2},\text{MAT},\alpha,\text{ade2},\text{his3-11,15},\text{leu2-3,112},\text{trp1-1},\text{ura3-1},\text{can},1-100$</td>
</tr>
</tbody>
</table>
smp3-1 /gaa1 - smp3-1 gaa1-2 MAT a ade2-1 ade3 Δ22 ura3-1 his3-11,15 trp1-1 can 1-100

GPI13

smp3-1/gpi13

pGAL-GPI13 gpi13::KAN R MAT a his3- Δ 1 leu2 Δ 0 lys2 met15 ura3 Δ 0

smp3-2/gpi13

pGAL-GPI13 smp3-2 gpi13::KAN R MAT a ade2-1 ade3 Δ 22 his3 met15 Δ 0 ura3
The essential Smp3 protein is required for addition of the side-branching fourth mannose during assembly of yeast glycosylphosphatidylinositols
Stephen J. Grimme, Barbara A. Westfall, Jill M. Wiedman, Christopher H. Taron and Peter Orlean

J. Biol. Chem. published online May 16, 2001

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