Gpi1, a *Saccharomyces cerevisiae* Protein That Participates in the First Step in Glycosylphosphatidylinositol Anchor Synthesis*

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Steven D. Leidich and Peter Orlean‡

From the Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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The temperature-sensitive *Saccharomyces cerevisiae gpi1* mutant is blocked in [3H]inositol incorporation into protein and defective in the synthesis of N-acetylgalcosaminylphosphatidylinositol, the first step in glycosylphosphatidylinositol (GPI) anchor assembly (Leidich, S. D., Drapp, D. A., and Orlean, P. (1994) J. Biol. Chem. 269, 10193–10196). The *GPII* gene, which encodes a 609-amino acid membrane protein, was cloned by complementation at the temperature sensitivity of *gpi1* and corrects the mutant's [3H]inositol labeling and enzymatic defects. Disruption of *GPII* yields viable haploid cells that are temperature-sensitive for growth, for [3H]inositol incorporation into protein, and for GPI anchor-dependent processing of the Gas1/Ggp1 protein and that lack in vitro N-acetylgalcosaminylphosphatidylinositol synthetic activity. The *GPII* protein thus participates in GPI synthesis and is required for growth at 37 °C. When grown at a semipermissive temperature of 30 °C, *gpi1* cells and *gpi1::URA3* disruptants form large, round, multiply budded cells with a separation defect. Homozygous *gpi1gpi1*, *gpi1::URA3gpi1::URA3, gpi2/ gpi2*, and *gpi3/gpi3* diploids undergo meiosis, but are defective in ascospore wall maturation for they fail to give the fluorescence due to the dityrosine-containing layer in the ascospore wall. These findings indicate that GPIs have key roles in the morphogenesis and development of *S. cerevisiae*.

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Glycosylphosphatidylinositols (GPIs),† which have the conserved core structure protein-CO-NH-CH₂-CH₂-POR- 6Mano1,2Mano1,6Mano1,4GlcNo1,6-myo-inositol-1-PO₄-lipid (1–5), anchor a variety of proteins to the external surface of the plasma membrane of eukaryotic cells. GPIs may also serve as sorting signals during intracellular protein transport by promoting the clustering of the proteins they anchor into membrane microdomains that become incorporated in specifically targeted vesicles (4). Glycolipid anchors on cell-surface receptors may allow such molecules to cluster with other proteins to complete a transmembrane signaling pathway (5, 6). GPIs are also important surface components of eukaryotic microbes:

they anchor the variant surface glycoprotein of trypanosomes (1, 2) and serve as intermediates in the transfer of glycoproteins from the yeast plasma membrane to cell wall glycans (7). GPIs are synthesized stepwise in the membrane of the endoplasmic reticulum beginning with the transfer of GlcNac from UDP-GlcNac to an acceptor phosphatidylinositol (1–3). The completed GPI precursor is then transferred to the COOH-terminal amino acid of target proteins in the lumen of the endoplasmic reticulum, after which these proteins transit the secretory pathway and become anchored in the plasma membrane.

Proteins involved in GPI anchoring are beginning to be identified through the isolation of GPI-anchoring mutants and the cloning of the normal counterparts of the affected genes (3). Mammalian cell lines defective in glycolipid anchoring have been identified on account of their inability to express GPI-anchored proteins on their cell surface (8–15). In humans, a GPI anchoring deficiency can lead to paroxysmal nocturnal hemoglobinuria (16, 17), which is due to a somatic mutation in the *PIG-A* gene (3, 18, 19). To study the biosynthesis and function of GPIs in a unicellular eukaryote, we devised a strategy to isolate GPI-anchoring mutants in *Saccharomyces cerevisiae* in which we screened colonies of mutagenized cells for those blocked in the incorporation of [3H]inositol into protein (20). The *gpi1*, *gpi2*, and *gpi3* mutants, identified in this way, are all temperature-sensitive for growth and for [3H]inositol incorporation into protein, and moreover, any pairwise combination of these three *gpi* mutations renders haploid cells incapable of vegetative growth. These findings indicate that GPIs are of key importance for the growth of *S. cerevisiae* (20, 21).

Studies with yeast and mammalian GPI-anchoring mutants indicate that the first step in GPI synthesis, the transfer of GlcNac from UDP-GlcNac to phosphatidylinositol, is unexpectedly complex. The yeast *gpi1*, *gpi2*, and *gpi3* mutants are all defective in GlcNac-PI synthesis (20, 21), and mutations in three mammalian genes (originally identified as the Thy-1 Class A, C, and H loci in mutant murine thymoma cells, respectively) also abolish GlcNac-PI synthesis (12, 13, 15). Among these mammalian and yeast mutants, only Thy-1A and *gpi3* cells are defective in obviously related proteins. Thus, the *Gpi3* protein (also known as Spt14 (22)) shows 44% identity to the human Pig-A protein at the amino acid sequence level (21, 23–25). The yeast *GPI2* gene has been cloned (21), but a counterpart of the human *GPI-H* gene (26) has so far not been found in the yeast genome. We report here the isolation of the *S. cerevisiae GPI1* gene and present evidence that its product participates in GPI synthesis, and we show that the GPI anchoring deficiency in *gpi* mutants affects morphogenesis and sporulation.

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**EXPERIMENTAL PROCEDURES**

Yeast Strains and Culture Media—Wild-type strains used were *S. cerevisiae* PP-1B (*MATa his4 chs1::URA3 ura3-52*), XM37-10C (*MATa ade2-101 ura3-52*), PRY238 (*MATa a ura3-52 leu3-112*), and PP-1D...
(MATa ade2-101 his4 ura3-52), gpi1 strains 2A and 8A (both MATa ade2-101 his4 ura3-52 his4) were derived from the third-fourth of the gpi1 mutation, respectively (20). Strain 11C (gpi1 MATa ura3-52 his4) was used for integrative mapping of the GPI1 locus. Strains L5686 (MATa ura3-52 his3:his2) and L5487 (MATa ura3-52 leu2:his3) were obtained from C. Styles and G. R. Fink. These two strains were crossed, yielding diploid L5686 × L5487. gpi1 disruptions were created in strains L5686 and L5487, and the two disruptants were crossed to give diploid L5686×L5487. Growth media were as described in Ref. 20. Diploids L5686 × L5487 and L5686 Δapp1 × L5487 Δapp1 were tested for their ability to form pseudohyphae on solid SLAHD medium (27).

[3H]Insitol Labeling of Protein—[3H]Insitol labeling of protein for subsequent separation by SDS-polyacrylamide gel electrophoresis was carried out using the dideoxy chain termination method according to the manufacturer's instructions. The ability of diploids and diploid Ura" transformants as described in Ref. 21. DNA was digested with EcoRI (6 units/μg), electophoresed through 1% agarose, transferred to nylon membranes, and then cross-linked to the membrane using a UV Stratalinker® 1800 (Stratagene) delivering 120,000 μJ of ultraviolet radiation. The probe used to confirm disruption of gpi1::URA3.1 was a 436-bp PstI-SacI fragment containing a portion of the 3' end of GPI1 (see Fig. 3A); that used for gpi1::URA3.2 was a 940-bp SacI-EcoRI fragment. Probes were labeled with digoxigenin-UTP using the Genius® random-primed DNA labeling and detection kit (Boehringer Mannheim). Membranes were prehybridized in a rotating oven at 65 °C for 2 h with 25 ml of 5 × SSC containing 0.1% (v/v) N-lauroylsarcosine, 0.02% (v/v) SDS. Digoxygenin-labeled DNA was added to the prehybridization solution at a concentration of 30 ng/ml and allowed to hybridize to the membranes overnight at 65 °C. Membranes were washed twice with 2 × SSC containing 0.1% SDS for 10 min at 65 °C and then twice with 0.5 × SSC and 0.1% SDS for 15 min at 65 °C. Hybridized DNA fragments were detected using anti-digoxigenin antiserum and Lumi-Phos® 530 (Boehringer Mannheim), following the manufacturer's instructions.

Disruption of the GPI1 Gene—The EcoRI fragment of genomic DNA in plasmid pSLG12 containing the GPI1 gene was excised by digestion with BamHI and XhoI, and the 3.0-kb fragment containing GPI1 was subcloned into the BamHI-XhoI site of pBluescript. This construct (pSLG1B1) contained four HincII sites, two of which were located 448 bp apart. The inositol coding region of the GPI1 gene was subcloned from adjacent sequences contributed by pBluescript vector and the genomic DNA flanking the GPI1 coding sequence, respectively. Removal of these two sites was necessary in order to allow deletion of the 448-bp internal HincII fragment. The HincII site present in the polynucleotide sequence was destroyed by digesting pSLG1B1 with SacI and XhoI, after which the plasmid was ligated together with T4 DNA ligase, yielding pSLG1B1. The HincII site present in the genomic DNA sequence was removed as follows. pSLG1B1 was digested with NcoI and NorI. The BamHI site present in the adjacent polynucleotide sequence of pBluescript, and the NorI sites (determined from partial sequence analysis) were located 5' to the internal HincII site. The fragments' 5' ends were made blunt-ended by filling in with the Klenow fragment of Escherichia coli DNA polymerase I and then joined with T4 DNA ligase to form pSLG1B2. The BamHI site is regenerated in this blunt-ended ligation. pSLG1B2 was digested with HindII, and a 1.1-kb HindIII fragment (blunt, Klenow) containing the URA3 gene was cloned into the gap that had been generated (see Fig. 3A), forming ppp1::URA3.1. A fragment containing the disrupted gene was excised from pSLG1B2 with EcoRV and BamHI and used to transform the Ura" haploid strain PP-1D and diploid strain PRY238.

For Southern blots, chromosomal DNA was isolated from untransformed haploid and diploid Ura" transformants as described in Ref. 21. DNA was digested with EcoRI (6 units/μg), electophoresed through 1% agarose, transferred to nylon membranes, and then cross-linked to the membrane using a UV Stratalinker® 1800 (Stratagene) delivering 120,000 μJ of ultraviolet radiation. The probe used to confirm disruption of gpi1::URA3.1 was a 436-bp PstI-SacI fragment containing a portion of the 3' end of GPI1 (see Fig. 3A); that used for gpi1::URA3.2 was a 940-bp SacI-EcoRI fragment. Probes were labeled with digoxigenin-UTP using the Genius® random-primed DNA labeling and detection kit (Boehringer Mannheim). Membranes were prehybridized in a rotating oven at 65 °C for 2 h with 25 ml of 5 × SSC containing 0.1% (v/v) N-lauroylsarcosine, 0.02% (v/v) SDS. Digoxygenin-labeled DNA was added to the prehybridization solution at a concentration of 30 ng/ml and allowed to hybridize to the membranes overnight at 65 °C. Membranes were washed twice with 2 × SSC containing 0.1% SDS for 10 min at 65 °C and then twice with 0.5 × SSC and 0.1% SDS for 15 min at 65 °C. Hybridized DNA fragments were detected using anti-digoxigenin antiserum and Lumi-Phos® 530 (Boehringer Mannheim), following the manufacturer's instructions.

Immediately after addition of 10 μl of a chase solution containing 0.3% (w/v) methionine, [3H]inositol labeling of protein for 30 min was started. Removal of these two sites was necessary in order to allow deletion of the 448-bp internal HincII fragment. The HincII site present in the polynucleotide sequence was destroyed by digesting pSLG1B1 with SacI and XhoI, after which the plasmid was ligated together with T4 DNA ligase, yielding pSLG1B1. The HincII site present in the genomic DNA sequence was removed as follows. pSLG1B1 was digested with NcoI and NorI. The BamHI site present in the adjacent polynucleotide sequence of pBluescript, and the NorI sites (determined from partial sequence analysis) were located 5' to the internal HincII site. The fragments' 5' ends were made blunt-ended by filling in with the Klenow fragment of Escherichia coli DNA polymerase I and then joined with T4 DNA ligase to form pSLG1B2. The BamHI site is regenerated in this blunt-ended ligation. pSLG1B2 was digested with HindII, and a 1.1-kb HindIII fragment (blunt, Klenow) containing the URA3 gene was cloned into the gap that had been generated (see Fig. 3A), forming ppp1::URA3.1. A fragment containing the disrupted gene was excised from pSLG1B2 with EcoRV and BamHI and used to transform the Ura" haploid strain PP-1D and diploid strain PRY238. A more extensive deletion of GPI1, which removes 83% of the gene's coding region, was introduced at the GPI1 locus using the γ-transformation technique (21, 37). A 2.3-kb EcoRV-EcoRI fragment of genomic DNA ending at the EcoRI site 31 bp upstream of the start site for the GPI1 gene and a SacI-BamHI fragment containing 264 bp of the GPI1 3'-sequence were subcloned in tandem, but in reverse order, in the SacI-BamHI and Smal-EcoRI sites, respectively, of the yeast integrating vector pRS406, creating a unique BamHI site between the two segments of GPI1 sequence. The EcoRV site in the EcoRV-EcoRI fragment was derived from the YEp24 vector sequence from which the fragment was excised. The BamHI site in the SacI-BamHI fragment was derived from the polylinker sequence in the plasmid from which it was excised. Construction of this plasmid, ppp1::URA3.2, resulted in the deletion of 1563 bp, including the start site, of the coding region of GPI1. Plasmid ppp1::URA3.2 was linearized by digestion with BamHI and used to transform the Ura" diploid strain 9933-13A into the plasmid region of GPI1. Plasmid ppp1::URA3.2 was linearized by digestion with BamHI and used to transform the Ura" diploid strain 9933-13A × XM37-10C. Ura" transformants were selected and analyzed by Southern blotting to confirm that the disrupted copy of GPI1 had been integrated at the expected chromosomal site. The organization of the chromosomal GPI1 locus following integration of ppp1::URA3.2 is shown in Fig. 3B. Disruptions of GPI1 in strains L5686 and L5487 were carried out with both the gpi1::URA3.1 and ppp1::URA3.2 constructs.

Microscopic Analysis—Wild-type and gpi1 cells were grown to log-arithmetic phase in liquid YEP medium (2% yeast extract/0.67% Bacto-peptone/2% glucose medium [YPD medium]) at 25 °C. Some 10³ cells from each culture were fixed and stained with either DAPI or Calcofluor white as described in Ref. 38. DAPI staining of nuclei in meiotic cells was performed according to Ref. 39. Cells were examined with a Zeiss Axiosplan fluorescence microscope at 1000 × magnification using both Nomarski-differential phase interference and fluorescence techniques. The ability of diploids to form...
RESULTS

Cloning of the GPI1 Gene—The wild-type counterpart of the defective gene in the gpi1 mutant was cloned by complementation of that mutant's temperature sensitivity with a yeast genomic DNA library in the 2μ plasmid YEp24. Plasmids were recovered from colonies of transformed gpi1 cells that grew at 37 °C, amplified in E. coli, and then retested for their ability to rescue the gpi1 cells' temperature sensitivity. One plasmid was isolated that complemented the gpi1 mutation upon reintroduction into the gpi1 strain. A 3.0-kb EcoRI fragment was excised from this plasmid (pGC-8.1) and subcloned into the 2μ vector pRS426, yielding pSLG12; this plasmid also corrected the gpi1 mutant's temperature sensitivity. No plasmids encoding potential multicopy suppressors of gpi1 were identified in this experiment.

gpi1 cells harboring pGC-8.1 were tested for correction of their GPI anchoring defects. Pulse labeling at 37 °C showed that [3H]inositol labeling of protein, which provides an indication of the cells' ability to carry out GPI anchoring, was restored in the transformed gpi1 cells (Fig. 1A, lanes 4 and 6). Furthermore, in vitro assay of membranes of gpi1 cells harboring pGC-8.1 showed that GlcNAc-PI synthetic activity was restored (Fig. 1B, lane 4). Introduction of the complementing DNA on a plasmid, a type of vector that often allows overexpression of the gene it contains, did not, however, lead to overproduction of GlcNAc-PI synthetic activity relative to the activity in control wild-type membranes (Fig. 1B, lane 2). We have not tested whether levels of Gpi1p are indeed elevated in control wild-type membranes (Fig. 1B, lane 2).

DNA Sequence Analysis—We sequenced the 3.0-kb genomic DNA fragment in pSLG12 that complemented the gpi1 mutant's temperature sensitivity (Fig. 2A) and subsequently found excellent agreement between our sequence and the sequence obtained in the course of the yeast genome sequencing project when the latter was deposited in the data base. These analyses revealed one large open reading frame of 1827 bp, encoding a predicted protein of 609 amino acids with a molecular mass of 70,308 Da (Fig. 2B). Comparisons of the Gpi1 protein sequence with those of other proteins in the data base using the BLASTP program (41) showed that the predicted amino acid sequence of Gpi1 has no similarity to any protein in the data base whose function is known, including the mammalian Pig-A and Gpi-H proteins. The latter is the product of a gene that complements the GlcNAc-PI synthesis-defective Thy-1 Class H lymphoma mutant (26). The Gpi1 protein also shows no resemblance to the S. cerevisiae Gpi2 protein.

To establish that the cloned yeast DNA contained the wild-type counterpart of the defective gene in the gpi1 mutant, the wild-type chromosomal sequence homologous to the DNA in the plasmid insert was marked with the URA3 gene. This was done using the integrating plasmid pSLG14, which contained the gpi1-complementing region and the URA3 gene. This plasmid was linearized by digestion with HpaI, which cleaves at a single site in GPI1, and introduced into the wild-type haploid strain XM37-10C to yield strains prototrophic for uracil as a result of integration of the URA3 gene at the chromosomal locus homologous to the plasmid's gpi1-complementing insert. One such haploid was then mated with a gpi1 ura3 haploid, and the resulting diploids were induced to sporulate, whereupon the ascI formed were submitted to tetrad analysis. All 20 tetrads analyzed yielded two Ura + segregants that grew at 37 °C and two Ura − segregants that were temperature-sensitive. This indicates that the putative GPI1 gene and the inte-

graded URA3 gene had become very tightly linked in the genome, a result that is strong evidence that the cloned gene is GPI1.

Yeasts GPI1 Gene and Morphogenetic Defects in gpi Mutants

Fig. 1. Correction of the GPI anchoring and GlcNAc-PI synthetic defects in gpi1 cells harboring the cloned GPI1 gene. A, restoration of [3H]inositol incorporation into proteins. Cultures of gpi1 8A cells (g1) harboring GPC-8-1 were grown at 25 °C and divided into two equal portions, one of which was shifted to 37 °C for 20 min. Shifted and control cultures were then radiolabeled with 100 μCi of [3H]inositol/ml for 40 min at 37 and 25 °C, respectively, after which radiolabeled proteins were extracted and separated by SDS-polyacrylamide gel electrophoresis, and [3H]-labeled proteins were made visible by fluorography. Cultures of wild-type (WT) cells and of gpi1 8A cells harboring the vector alone (V) were radiolabeled in parallel as controls. B, restoration of in vitro GlcNAc-PI synthetic activity in transformants. Mixed membranes prepared from the strains used in the experiment in A were assayed for the synthesis of [14C]GlcNAc-PI, [14C]GlcN-PI, and [14C](GlcN-acetyl)inositol-PI. Incubations were for 10 min. Assay conditions and the procedures for the isolation, chromatographic separation, and detection of [14C]-labeled lipids were as detailed in Ref. 30. A sample of lipids from wild-type cells labeled in vivo with [3H]inositol was run in lane 1 of B.

3 C. Taron, B. Westfall, and P. Orlean, unpublished data.

4 P. Colussi, C. Taron, P. Orlean, unpublished data.
likely transmembrane segments (Fig. 2C), consistent with the fact that in vitro GlcNAc-PI synthetic activity sediments with mixed membranes (20, 30). The predicted Gpi1 protein also contains three sites to which an N-linked oligosaccharide could become attached, but the protein’s NH2 terminus does not meet the criteria for a signal sequence. A potential site for tyrosine autophosphorylation, Arg-Asn-Arg-Ile-Asp-His-Asn-Tyr (residues 353–360), was identified using the Prosites software package (43), but no evidence could be obtained that Gpi1p can be phosphorylated at this site in vivo, either in vegetative cells or under other conditions tested. For example, MATα haploids were treated with α-mating pheromone, and Western blots of cellular proteins were probed with anti-phosphotyrosine antibodies, but no cross-reacting protein was seen in the size range predicted for Gpi1p, although the expected 40-kDa tyrosine-phosphorylated protein (44) was present. Furthermore, cells grown in lactate medium (45), from which a GPI-anchored protein can be isolated (46), revealed no tyrosine-phosphorylated Gpi1p.

Disruption of the GPI1 Gene—The GPI1 gene was disrupted by deleting 448 base pairs of its DNA (25% of the coding region) and replacing them with the URA3 gene to create the...

Fig. 2. Cloning of the GPI1 gene. A, subcloning of the yeast genomic DNA insert that complements the gpi1 mutation. B, DNA and deduced amino sequences of the GPI1 gene. The putative tyrosine autophosphorylation site is underlined. C, hydropathy profile of the Gpi1 protein (35, 42).
haploid segregant.

Representative ascus obtained after sporulation of the chromosomes are 3.0 and 3.6 kb, respectively. Dip DNA containing the 3

brane, the DNA was probed with a digoxigenin-labeled fragment of Following agarose gel electrophoresis and transfer to a nylon mem-

diploid. Chromosomal DNA was isolated and digested with 

GPI1 XMI37-10C. Chromosomal DNA was isolated from the heterozygous 
gpi1::URA3.1 haploid strain PP-1D was disrupted with 

gpi1::URA3.1 construct (Fig. 3A), which was used to transform diploid and haploid strains to uracil prototrophy. Southern blot analysis confirmed that the gpi1::URA3 fragments had integrated at the correct chromosomal locus. Tetrad analysis of the meiotic segregants produced upon sporulation of the heterozygous GPI1/gpi1::URA3.1 diploids yielded four viable haploids. Likewise, integration of the gpi1::URA3.1 construct at the GPI1 locus of wild-type haploids also yielded viable cells. The recovery of viable gpi1::URA3.1 haploids indicated that GPI1 is not essential for yeast viability; however, the gpi1 disruptants were temperature-sensitive for growth. In the case of the disruption in the haploid strain PP-1D, Ura− transformants arising from integration of the gpi1::URA3.1 construct were crossed with the wild-type Ura+ strain XM37-10C. Heterozygous GPI1/gpi1::URA3.1 diploids were induced to sporulate, and the four haploid ascospores in the resulting asci were

Disruption of the GPI1 gene. A, construction of gpi1::URA3.1. The 448-bp HincII fragment was replaced with DNA containing the selectable marker URA3, and the linear EcoRI-BamHI fragment containing gpi1::URA3.1 was used to transform diploid and haploid S. cerevisiae strains as detailed under “Experimental Procedures.” B, genomic organization of the GPI1 locus following integration of gpi1::URA3.2. Plasmid gpi1::URA3.2 was constructed as described under “Experimental Procedures” and then introduced into the genome of S. cerevisiae strains using the γ-transformation procedure described in Refs. 21 and 37. C, Southern blot analysis to confirm the integration of gpi1::URA3.1 at the chromosomal GPI1 locus. The GPI1 gene in the haploid strain PP-1D was disrupted with gpi1::URA3.1, and the resulting gpi1::URA3.1 strain was crossed with the wild-type haploid strain XM37-10C. Chromosomal DNA was isolated from the heterozygous GPI1/gpi1::URA3.1 diploid and from the haploid progeny from a representative ascus obtained after sporulation of the GPI1/gpi1::URA3.1 diploid. Chromosomal DNA was isolated and digested with EcoRI. Following agarose gel electrophoresis and transfer to a nylon membrane, the DNA was probed with a digoxigenin-labeled fragment of DNA containing the 3′-portion of the GPI1 gene. The expected sizes of the hybridizing EcoRI fragments from the GPI1 and gpi1::URA3.1 chromosomes are 3.0 and 3.6 kb, respectively. Dip, heterozygous GPI1/gpi1::URA3.1 diploid; WT, GPI1 haploid segregant; Δ, gpi1::URA3 haploid segregant.

Growth and biochemical phenotypes of gpi1::URA3 cells. A, temperature-sensitive growth of gpi1::URA3.1 haploid segregants. The haploid progeny from the ascus obtained upon sporulation of the GPI1/gpi1::URA3.1 diploid used in the experiment in Fig. 3C were tested for growth on YPD medium at 25 and 37 °C. WT, wild-type haploid; Δgpi1, gpi1::URA3.1 haploid. B, temperature-sensitive incorporation of [3H]inositol into protein in gpi1::URA3.1 strains. Haploid segregants from the representative tetrads examined in Fig. 3C and in A were radiolabeled with [3H]inositol at 25 °C or after shift to 37 °C, after which [3H]inositol-labeled proteins were extracted and separated by SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 1. Δ, gpi1::URA3.1 haploid. C, defective maturation of the Gas1/Ggp1 protein in gpi1::URA3.1 strains. gpi1::URA3.1 (Δgpi1) cells were pulse-labeled at 25 or 37 °C, and wild-type (WT) and gpi1 cells were pulse-labeled at 37 °C with Tran35S-label mixture, after which radioactivity was chased with unlabeled amino acids as detailed under “Experimental Procedures.” 35S-Labeled Gas1p/Ggp1p was then immunoprecipitated from extracts of the cells, separated by SDS-polyacrylamide gel electrophoresis, and detected by fluorography. P, 30, and 60 indicate Gas1p/Ggp1p immunoprecipitated immediately after pulse labeling or after a 30- or 60-min chase period, respectively. D, gpi1::URA3.1 haploid segregants lack in vitro GlcNac-PI synthetic activity. Lysates (28) of the haploid segregants analyzed in B above were incubated with UDP-[14C]GlcNac, and [14C]GlcNac-PI, [3H]GlcN-PI, and [3H]GlcN-(acyliminositol)-PI (GlcN-(acyl-Ins)PI) were extracted and separated by TLC.
allele (Fig. 3C). The gpi1-disrupted haploids, however, grow more slowly than their wild-type siblings do at 25 °C, with their doubling time in YPD medium being 240 min compared with 150 min for the Gpi1+ strains.

An additional disruption in which a larger portion of the GPI1 gene was deleted (Fig. 3B) was subsequently done to rule out the possibility that a functional but truncated form of Gpi1p is encoded by the residual GPI1 sequence in the gpi1::URA3.1 construct. This additional construct, gpi1::URA3.2, was used to transform the wild-type diploid strain Y933-13A × XM37-10C. Gpi1+ transformants were selected and analyzed as described above. In the 20 tetrad analyzed, all four spores were viable, but the ura3 prototrophs again were temperature-sensitive.

The GPI2 gene, when present on a 2µ plasmid, was previously shown to suppress the gpi1 mutant's temperature-sensitive growth defect (21). GPI2 also suppresses the gpi1::URA3.1 strain's temperature sensitivity, but does so more weakly than it suppresses the temperature sensitivity of the gpi1 point mutant.

GPI Anchoring Defects in gpi1::URA3 Cells—The results of three different types of experiments indicate that gpi1-disrupted haploids are defective in GPI anchoring. First, gpi1::URA3.1 segregants and their Gpi1+ siblings from a representative ascus were tested for their ability to incorporate [3H]inositol into protein at 25 °C and after shift of the cultures to 37 °C. Pulse labeling of yeast cells with [3H]inositol provides an indication of their ability to carry out GPI anchoring because all detectable protein-linked [3H]inositol is present in GPI anchors (47). The gpi1::URA3.1 disruptants were blocked in the incorporation of [3H]inositol into protein at 37 °C and, by this criterion, are defective in GPI anchoring. gpi1::URA3.1 cells, however, clearly incorporated [3H]inositol into protein at 25 °C (Fig. 4B, lanes 1, 2, 7, and 8), indicating that disruption of the GPI1 gene does not lead to a complete abolition of GPI anchoring in the viable gpi1::URA3.1 cells. However, densitometric analysis of fluorographs of the proteins in GPI1 and gpi1::URA3.1 cells radiolabeled with [3H]inositol at 25 °C revealed that levels of [3H]inositol incorporated into protein by gpi1::URA3.1 cells were, on average, 40% of the levels incorporated by their wild-type siblings.

The second test for a GPI anchoring deficiency was based on the expectation that if gpi1 disruptants were blocked in GPI anchoring, they would be defective in the efficient transport and subsequent processing of the Gas1/Ggp1p protein. Abolition of GPI attachment causes this protein to remain in an immature, core-glycosylated 105-kDa form, rather than be transported to the Golgi apparatus, where it is normally converted to a more extensively glycosylated 125-kDa species (48). To examine the maturation of Gas1p/Ggp1p, wild-type, gpi1::URA3.1, and gpi3 cells were pulse-labeled at 25 or 37 °C with a mixture of [35S]methionine and [35S]cysteine, after which a chase period was initiated upon addition of an excess of unlabeled methionine and cysteine. [35S]Labeled Gas1p/Ggp1p was immunoprecipitated from cells harvested immediately after pulse labeling or after 30 and 60 min of chase. After pulse labeling and a 60-min chase at 37 °C, Gas1p/Ggp1p appeared in its mature 125-kDa form in wild-type cells (Fig. 4C, lane 1). In gpi1::URA3.1 cells at 25 °C, only the immature 105-kDa form of Gas1p/Ggp1p was detectable immediately following pulse labeling (Fig. 4C, lanes 2 and 5), but the mature 125-kDa form appeared during the subsequent chase period at 25 °C (lanes 3 and 4). In contrast, when the pulse and subsequent chase were carried out at 37 °C, Gas1p/Ggp1p synthesized in gpi1::URA3.1 cells remained in the 105-kDa form (Fig. 4C, lanes 5–7). As a control, gpi2 cells, which are defective in GlcNAc-PI synthesis (21, 25) and in which Gas1p/Ggp1p maturation is abolished at 37 °C (25), were pulse-chase-labeled at 37 °C. As expected, Gas1p/Ggp1p remained in its immature form (Fig. 4C, lane 9). gpi1::URA3.1 cells, like the previously described GPI anchoring-defective gpi3/spt14 cells (25), are therefore defective at non-permissive temperature in GPI anchor-dependent transport and processing of the Gas1/Ggp1p protein.

In our third test for GPI anchoring defects, we established that gpi1::URA3.1 segregants grown at 25 °C lack in vitro GlcNAc-PI synthetic activity (Fig. 4D, lanes 1 and 4), as is the case with the gpi1 point mutant grown at permissive temperature. Likewise, gpi1::URA3.2 disruptants synthesize no GlcNAc-PI in vitro (data not shown). From the biochemical and growth defects of gpi1::URA3 cells, we conclude that the Gpi1 protein participates in GPI anchoring and that it is required for the growth of S. cerevisiae cells at 37 °C.

Selective Effects of gpi Mutations on Yeast Growth and Development—We used our gpi mutants, which show lowered levels of GPI anchoring when grown at permissive temperatures, to explore the effects of a partial GPI anchoring deficiency on vegetatively growing cells and on the developmental processes of mating and sporulation and the formation of pseudohyphae. gpi1, gpi1::URA3.1, and gpi2 cells were examined by phase-contrast and fluorescence microscopy. When grown at 25 °C, gpi1::URA3.1 cells had a doubling time of ~240 min and resembled Gpi1+ cells in their morphology, but when grown at a semipermissive temperature of 30 °C, the doubling time increased to 640 min, and the cells were large, round, and multiply budded. Cells with unseparated buds, which had themselves initiated budding, were also present in the culture (Fig. 5). The gpi1 and gpi2 point mutants exhibited similar morphologies at 30 °C. These phenotypes resemble those of cells in which the GPI-anchored cell-surface glycoprotein Gas1/ Ggp1 has been deleted, although in contrast to gpi1::URA3.1 cells, gpi1 disruptants have a doubling time of 155 min (49). On many of the multiply budded gpi cells, the projection of...
growth of one bud was 90–180° relative to other bud(s). This suggests an alteration in the cells’ normal pattern of bud placement since wild-type haploids, including the parents of the strains used in these experiments, normally bud in an axial pattern and project new bud growth next to the previous site of budding (50–52). The alterations in bud placement were confirmed by Calcofluor white staining of chitin-containing bud scars and division septa in gpi1 and gpi1::URA3 cells; the scars on the cells’ surface were not always clustered at one cortex of the cell, but, in some cases, were located randomly on the surface (data not shown).

Since mutations in Gpi1p affect morphogenesis, we tested the gpi1 point and gpi1::URA3 deletion mutants for suppression of their temperature-sensitive growth by the SSD1-v1 allele of the polymorphic SSD1/SRK1 gene on low copy centromere-based vectors. SSD1-v1 suppresses mutations in a number of genes involved in growth control and morphogenesis in yeast (53–56), and the Ssd1 (Srk1) protein may be involved in protein phosphatase function (54). As a control, a Δacsd4 Sup’ strain (57) that is temperature-sensitive for growth and whose defect is suppressed by the SSD1-v1 gene was transformed in parallel. SSD1-v1 did not suppress the temperature sensitivity of the gpi1 point mutant and gpi1::URA3 strains, but did allow growth of the Δacsd4 Sup’ control.

We also tested gpi1 haploids for defects in mating. α-Mating agglutinin is GPI-anchored, and α-agglutinin contains a COOH-terminal peptide sequence that could serve as a signal for GPI attachment (58, 59), raising the possibility that the efficiency of mating between two haploid gpi1 mutants may be lowered due to reduced surface expression of these α-agglutinins, which contribute to cell-cell adhesion during the mating process. This was tested at 25 °C in liquid medium by mixing pairwise combinations of gpi1::URA3 MATa and gpi1::URA3 MATα strains with one another or with wild-type tester strains of opposite mating type. Numbers of diploids formed by gpi1 haploids were compared with control values obtained by mixing Gpi+ MATa and Gpi- MATα haploids, whose numbers were then normalized to 1.0 (60). Where one haploid partner was disrupted at the GPI1 locus, mating efficiencies were 0.70–0.74 relative to Gpi+ controls, whereas gpi1::URA3 MATa and gpi1::URA3 MATα strains mated with one another at efficiencies of 0.45–0.50. Since disruption of the AGR1 agglutinin gene results in mating efficiencies of $-1 \times 10^{-5}$ relative to wild-type values (60) and since the slower growth rate of gpi1::URA3 cells may in part contribute to the lower mating efficiencies observed, we conclude that disruption of GPI1 does not severely impair mating.

Homozygous gpi1 diploids show a severe sporulation defect. When incubated on solid or in liquid sporulation medium, gpi1/ gpi1 and gpi1::URA3/gpi1::URA3 diploids form no detectable ascospores, and gpi1/gpi2 and gpi1/gpi3 diploids form very few ascii containing any visible ascospores. However, the diploids had indeed responded to nutrient starvation on sporulation medium, for DAPI staining of the cultures revealed the presence of multiple nuclei in many of the cells, indicating that the cells had undergone meiosis, which precedes ascospore formation and maturation (Fig. 6A). Further evidence for defects in ascospore formation was obtained by qualitative assay of the diploids for the presence of dityrosine, a naturally fluorescing molecule that cross-links peptide chains in the outermost layer of the ascospore wall (61). Homozygous gpi1 diploids failed to emit fluorescence upon exposure to ultraviolet light, whereas wild-type diploids that had been incubated in parallel on sporulation medium fluoresced intensely (Fig. 6B). Dityrosine fluorescence is abolished in the dit1 and dit2 mutants, which are defective in steps in the conversion of L-tyrosine to d-L-tyrosine (40, 62), and is also abolished in chitin-synthesis-deficient dit101/csd2 mutants, which fail to make the chitosan that underlies the outer dityrosine layer (63). Since homozygous dit1 and dit2 diploids sporulate and form visible ascospores and since homozygous dit101 diploids also yield viable spores, the defect in gpi1 diploids may affect ascospore wall maturation at a stage earlier than formation of the outer dityrosine layer.

Upon starvation for nitrogen, certain S. cerevisiae diploids undergo a dimorphic transition to a pseudohyphal growth form in which cells become elongated and show uninuclear budding such that chains of cells are extended away from a colony (27). We tested whether a diploid strain that is normally capable of forming pseudohyphae could do so after its two copies of GPI1 had been disrupted. To do this, the GPI1 gene was disrupted in strains L5487 MATa and L5686 MATa, haploids that when crossed yield a diploid that can undergo the dimorphic transition. Both gpi1::URA3.1 and gpi1::URA3.2 disruptants were created. Two disruptants were mated, and the resulting homozygous gpi1::URA3/gpi1::URA3 diploids were tested for their ability to convert to pseudohyphae at 25 and 30 °C. The gpi1::URA3/gpi1::URA3 disruptants formed pseudohyphae at both temperatures, and the GPI1 gene is therefore not required for dimorphic transition in S. cerevisiae diploids.

**DISCUSSION**

The gpi1 point mutant is temperature-sensitive for growth and for GPI anchoring and is defective in vitro in GlcNAc-PI synthesis, the first step in GPI assembly (20). We have exploited this mutant’s temperature sensitivity to clone the wild-type GPI1 gene. GPI1 is not an essential gene, but the biochemical phenotypes of gpi1-disrupted cells show clearly that the
Gpi1 protein is involved in GPI anchoring. Thus, gpi1 disruptants are temperature-sensitive for growth, for [3H]inositol incorporation into protein, and for GPI anchor-dependent transport and processing of a model protein, and the gpi1 null mutants also lack in vitro GlcNAc-PI synthetic activity. A role for Gpi1p in GPI anchoring is also indicated by the fact that the combination of gpi1 with either the gpi2 or gpi3 mutations, which also affect GlcNAc-PI synthesis, is synthetically lethal (21). Although a functional Gpi1 protein is required for both GPI anchoring and for cell viability at 37°C, the finding that gpi1 disruptants are viable raised the question whether such cells carry out any GPI anchoring at all at their permissive temperature. They clearly do, for at 25°C, [3H]inositol is incorpo- rated into protein, and the Gas1/Ggp1 protein is converted to its mature form in gpi1::URA3.1 cells.

Gpi1p shows no resemblance to yeast Gpi2p or to the human Pg-A and Gpi-H proteins, all of which are involved in GlcNAc-PI synthesis. The murine Thy-1 Class C lymphoma mutant is also blocked in GlcNAc-PI synthesis, but whether these cells are defective in a mammalian counterpart of yeast Gpi1p or Gpi2p is not yet known. At least four proteins may therefore be involved in GlcNAc-PI synthesis in eukaryotes, although their functions have yet to be established. Of these proteins, Gpi3p/Pig-A is the only obvious candidate for the catalytic subunit insofar as its sequence shows similarity to certain glycosyltransferases (23, 24). It has been speculated that the proteins implicated in GlcNAc-PI synthesis form part of a complex (1, 21, 25), and a possible role for nonessential Gpi1p may be to stabilize such a protein assembly, a function required at high temperature in vivo, and for enzymatic activity in vitro. The participation of multiple proteins in one step in GPI synthesis has its parallel in the yeast oligosaccharyltransferase, which is a complex of at least six proteins, one of which, Ost3p, is nonessential for viability (64).

An alternative function for Gpi1p is as a regulator of expression of genes involved in GPI synthesis. However, Gpi1p is a membrane protein, contains no known DNA-binding motifs, and shows no resemblance to transcriptional activators. Furthermore, a role for Gpi1p as a transcriptional regulator is difficult to reconcile with the fact that membranes prepared from gpi1 cells grown at permissive temperature lack detectable in vitro GlcNAc-PI synthetic activity. If the Gpi1p made in the gpi1 point mutant were a thermolabile regulator of expression of genes encoding the GlcNAc-PI synthetic proteins, we would expect the latter proteins to be made at permissive temperature and their presence to be detectable by in vitro enzyme assay.

The phenotypes of the GlcNAc-PI synthesis-defective gpi mutants indicate that GPIs have selected but key roles in yeast growth and development and that these structures are required for normal cell wall growth and budding in vegetative cells and for ascospore wall maturation during sporulation. On the other hand, diminished GPI synthesis in homozygous gpi1::URA3/gpi1::URA3 diploids does not affect the ability of these strains to form chains of pseudohyphal cells, indicating that GPIs have no significant role in this polarized growth process.

The abnormal formation and expansion of the cell wall in vegetatively growing gpi1 and gpi1::URA3 cells at 30°C may be due to a failure in cell-surface delivery of key proteins that are normally GPI-anchored. Indeed, at least one GPI-anchored protein, Gas1/Ggp1/Cwsh52, is required for normal levels of synthesis of cell wall β-1,3-glucan (65). Furthermore, a perturbation in GPI anchoring would result in an inability of certain cell wall glycoproteins to be transferred from a GPI-anchored, plasma membrane-bound intermediate to a cell wall acceptor glucan (7). Such effects of a GPI deficiency are consistent with the finding that mutations allelic to gpi3 (cuh6) cause defects in cell wall assembly that render cells hypersensitive to Calcofluor white (24). In addition to or as a consequence of these cell-surface assembly defects, inefficient GPI anchoring in gpi mutants may cause proteins that participate in bud placement to be mislocalized to abnormal sites on the cell surface, resulting in deviations from the normal axial bud placement pattern.

Homozygous gpi1/gpi1 diploids fail to form refractile ascospores and to make a dityrosine-containing ascospore wall, but the exact stage in ascospore development that is affected is not known. Although refractile ascospores are not visible in gpi1/gpi1 diploids by phase-contrast microscopy, thin section electron micrographs may yet reveal the presence of incomplete or aberrant ascospore walls in these cells. Mutations or treatments affecting the synthesis of other GlcN-containing ascos- pore wall components give phenotypes similar to those of gpi1/gpi1 diploids. Thus, homozygous gen1/gen1 glucosamine auxotrophic diploids form multinucleate ascospores on sporulation medium, but fail to make refractile spores that are visible by phase-contrast microscopy; however, thin section electron microscopy shows the presence of ascospores that lack their two outer layers (66). Inhibition of asparagine-linked glycosylation with tunicamycin likewise does not prevent meiosis, and although refractile ascospores are not formed, electron microscopy reveals spores lacking the outer wall layer (67). These findings, together with the severe sporulation defect in homozygous gpi1/gpi1 diploids at permissive temperature, indicate key roles for the Gpi1, Gpi2, and Gpi3 proteins in ascospore wall maturation. In these gpi1/gpi1 diploids, GPIs are presumably made at a level below a threshold needed for ascospore wall maturation. Protein(s) that need to be GPI-anchored in order for ascospores to mature have not, however, been identified. Although homozygous gpi1/gpi1 diploids have a severe sporulation defect, this does not explain the double mutant lethality observed when any pairwise combination of the gpi1, gpi2, and gpi3 mutations is present in the same haploid strain (21). Thus, diploids formed by crossing gpi1 and gpi2, gpi1 and gpi3, and gpi2 and gpi3 haploids form four ascospores that are all capable of germinating. However, those haploids inferred to be double gpi mutants proceed through no more than five rounds of cell division before ceasing vegetative growth.4

The yeast gpi mutants and genes we have isolated reveal the complexity of the first step in GPI synthesis and highlight the roles GPIs play in yeast growth and development. Gpi1p is of further significance: it is required for growth of S. cerevisiae at 37°C, and should this protein have a similar function in fungal pathogens, it may then be required for growth of such organisms in their human host. Furthermore, our finding that gpi1-disrupted haploids are viable allows us to screen for mutants that require the GPI1 gene for viability. Given that double gpi mutants are incapable of vegetative growth (21), mutations in additional genes involved in GPI anchoring may be among those showing synthetic lethality with GPI1.

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