

# Identification of Six Complementation Classes Involved in the Biosynthesis of Glycosylphosphatidylinositol Anchors in *Saccharomyces cerevisiae*

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**Abstract.** Glycosylphosphatidylinositol (GPI)-anchored membrane proteins are synthesized by the posttranslational attachment of a preformed glycolipid to newly made glycoproteins.  $\alpha$ -Agglutinin is a GPI-anchored glycoprotein that gets expressed at the cell surface of *MAT $\alpha$*  cells after induction with type a mating factor. Mutants affecting the biosynthesis of GPI anchors were obtained by selecting for the absence of  $\alpha$ -agglutinin from the cell wall after induction with a-factor at 37°C. 10 recessive mutants were grouped into 6 complementation classes, *gpi4* to *gpi9*. Mutants are considered to be deficient in the biosynthesis of GPI anchors, since each mutant accumulates an abnormal, incomplete GPI glycolipid containing either zero,

two, or four mannoses. One mutant accumulates a complete precursor glycolipid, suggesting that it might be deficient in the transfer of complete precursor lipids to proteins. When labeled with [2-<sup>3</sup>H]inositol, mutants accumulate reduced amounts of radiolabeled GPI-anchored proteins, and the export of the GPI-anchored Gas1p out of the ER is severely delayed in several mutant strains. On the other hand, invertase and acid phosphatase are secreted by all but one mutant. All mutants show an increased sensitivity to calcofluor white and hygromycin B. This suggests that GPI-anchored proteins are required for the integrity of the yeast cell wall.

**M**ANY glycoproteins of lower and higher eukaryotes are attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI)<sup>1</sup> anchor covalently linked to their carboxy terminus (15, 41). These anchors have the conserved core structure protein-CO-NH-(CH<sub>2</sub>)<sub>2</sub>-PO<sub>4</sub>-6Man $\alpha$ 1-2Man $\alpha$ 1-6Man $\alpha$ 1-4GlcNH<sub>2</sub>-*myo*-inositol-PO<sub>4</sub>-lipid. Anchors are synthesized by stepwise addition of sugars and phosphoethanolamine to phosphatidylinositol (PI) to form the complete precursor (CP) glycolipid. The transfer of CP's onto proteins is effected by a yet unidentified enzyme complex that recognizes a GPI signal sequence in the COOH-terminal 30–40 amino acids of GPI proteins. This enzyme complex cleaves off most of the GPI signal sequence and replaces it with the preassembled CP. Although all biosynthetic reactions required for GPI an-

chor biosynthesis have been reproduced in microsomal in vitro systems and are quite well understood (11, 13, 24, 25, 37–40, 42, 43, 45), the identification and study of individual enzymes are still far from complete. Four genes required for GPI biosynthesis have been cloned by complementation of mammalian somatic cell mutants deficient in the biosynthesis of CP's (29–31, 46). These mutant cell lines were isolated by selecting for the loss of surface expression of GPI-anchored proteins, and show normal growth in tissue culture (28). Of the four genes isolated by complementation cloning, two are required for the addition of GlcNAc to PI (30, 46), one for the addition of the third, i.e., the  $\alpha$ 1,2-linked mannose (31), and one for the addition of phosphoethanolamine (29). In contrast to mammalian cell lines, GPI anchoring seems to be essential for yeast cells (33). Nevertheless, several conditional *Saccharomyces cerevisiae* mutants have been obtained in a screen for the absence of [2-<sup>3</sup>H]inositol incorporation into proteins at 37°C (33, 56). Mutants belonging to three complementation classes were deficient in the addition of GlcNAc to PI, and one other mutant did not add mannose to the GlcN-PI intermediate. To obtain additional yeast mutants, we tried to develop a selection for GPI biosynthesis mutants in yeast.

*S. cerevisiae* contains numerous GPI proteins, some of which are very highly glycosylated, but the function of

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1. *Abbreviations used in this paper.* CFW, Calcofluor white; CP, complete precursor; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D from bovine serum; HF, hydrofluoric acid; Ins, *myo*-inositol; JBAM, jack bean  $\alpha$ -mannosidase; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; ts, temperature sensitive; [2-<sup>3</sup>H]Ins, [2-<sup>3</sup>H] *myo*-inositol.

most of them is still unknown (7). The biosynthesis and intracellular transport of two GPI proteins, namely Gas1p and  $\alpha$ -agglutinin, have been studied in some detail (17, 36, 58, 74). Proteins receive their anchor in the ER (10), and are supposed to be transported to the Golgi apparatus by vesicular traffic, since this transport is controlled by the same genes as the transport of soluble secretory proteins (7, 14, 59, 67). Additionally, ER to Golgi apparatus transport of Gas1p has been shown to depend on ongoing sphingolipid biosynthesis (26). Whereas Gas1p, as well as another GPI protein, cAMP-binding protein, are destined to stay at the plasma membrane (17, 48, 49, 54, 58),  $\alpha$ -agglutinin loses the inositolphospholipid moiety after having reached the plasma membrane, and appears at the outer surface of the cell wall (36). The mature  $\alpha$ -agglutinin becomes covalently linked to the  $\beta$ -glucans of the cell wall (35, 47, 75).  $\alpha$ -Agglutinin has been shown to depend critically on its GPI anchor for integration into the cell wall, since removal of the COOH-terminal hydrophobic GPI signal sequence resulted in the loss of cell-surface attachment, and allowed efficient secretion of the truncated  $\alpha$ -agglutinin (69, 80). On the other hand, introduction of mutations in the COOH-terminal GPI signal sequence of Gas1p renders the addition of a GPI in the ER impossible, and results in the ER retention of the unprocessed GPI protein (53). It thus can be expected that mutants deficient in the biosynthesis of the GPI glycolipid or its transfer to proteins would either retain the unprocessed  $\alpha$ -agglutinin in the ER, or would secrete fragments thereof, and would therefore be deficient in the expression of  $\alpha$ -agglutinin at the cell surface. Here we describe the results of a selection procedure based on this assumption.

## Materials and Methods

### Strains, Growth Conditions, and Materials

*S. cerevisiae* strains were W303-1B (*MAT $\alpha$  ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11, 15*) and W303-1A (*MAT $\alpha$  ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11, 15*), X2180-1A (*MAT $\alpha$* ), HMSF176 (*MAT $\alpha$  sec18-1*), C4 (*MAT $\alpha$  leu2-3, 112 ura3-52 pmi40*) and RH932 (*MAT $\alpha$  end2-1 leu2 ura3 bar1-1*). Cells were kept on yeast extract/peptone/dextrose (YPD) plates (9), and were cultured in salt/dextrose (SD) medium containing salts, vitamins (but no *myo*-inositol [Ins]), trace elements, and 2% glucose as a carbon source; SDC medium is the same, but in addition contains 1% casein hydrolysate. SDUA and SDCUA are SD and SDC media supplemented with uracil and adenine, each at 40  $\mu$ g/ml. The OD of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm; 1 OD<sub>600</sub> U of cells corresponds to 1–2.5  $\times 10^7$  cells, depending on the strain. Reagents were obtained from the following sources: anti-rabbit-IgG/alkaline phosphatase conjugate (A2556) from Sigma Chemical Co. (St. Louis, MO); Amberlite XAD-2 resin from Serva Fine Biochemicals (Heidelberg, Germany); Dynabeads M-280 coupled to sheep anti-rabbit-IgG from Milan Analytical AG (La Roche, Switzerland); EN<sup>3</sup>HANCE from NEN (Hertfordshire, UK); 0.2-mm-thick silica gel 60 plates (20  $\times$  20 cm) from Merck (Darmstadt, Germany); mixed bed ion exchange resin (AG-501-X8; Bio-Rad Laboratories, Richmond, CA) ammonia in methanol (7 M; Janssen Chimica, Beerse, Belgium); phosphatidylinositol-specific phospholipase C (PI-PLC) from Boehringer Mannheim GmbH (Mannheim, Germany). GPI-phospholipase D (PLD) purified 1,000-fold from bovine serum was a kind gift of Dr. U. Brodbeck, University of Bern, Bern, Switzerland.

$\alpha$ -Factor was isolated by incubation of cells with Amberlite XAD-2 resin, and quantitated as described (73). A polyclonal antiserum was raised against  $\alpha$ -agglutinin in a rabbit by repeated s.c. and i.v. injection as described (79).

### Isolation of GPI-deficient Mutants

Mutants were obtained using the scheme depicted in Fig. 2. Ethyl meth-

anesulfonate mutagenesis was performed as described (34), and cells were allowed to recover by an overnight incubation at 24°C in SDCUA, buffered to pH 5.0 with 0.05 M phosphate. Before immunoselection, 3 ml of Dynabeads M-280 suspension (6–7  $\times 10^8$  beads per ml) were prewashed, once with 25 ml PBS, twice with 25 ml PBS containing 5% FCS, and once with 25 ml PBS containing 1% FCS (PBSF), and finally resuspended in 15 ml of PBSF. 10<sup>8</sup> mutagenized cells were resuspended in 10 ml SDCUA buffered with 0.05 M phosphate to pH 5; cells were preincubated for 15 min at 37°C, and then stimulated with  $\alpha$ -factor (20 U/ml, final concentration) for 90 min at 37°C. The cells were then washed with 25 ml PBSF at 37°C, resuspended in 4.5 ml PBSF, and 0.5 ml anti- $\alpha$ -agglutinin rabbit antiserum was added. Cells were incubated 90 min at 37°C on a slowly rotating wheel. Cells were then washed five times with 20 ml of PBSF, and resuspended in 10 ml PBSF. For the first round of immunoselection, 5 ml of prewashed Dynabeads (coated with sheep anti-rabbit-IgG antibodies) was added to the cells, and was incubated with the cells for 90 min at 24°C on a wheel. The tube was then placed for 5 min against the magnet, and the medium with the unbound cells was collected in a recipient vessel. At this stage, the magnetic beads were washed five times, by each time removing the tube from the magnet, resuspending beads in fresh PBSF (15 ml), placing the tube next to the magnet for 5 min, and collecting the free cells into the recipient vessel. These washings were necessary to recover the cells that display a normal, constitutive expression of  $\alpha$ -agglutinin at 24°C. Cells pooled in the recipient vessel were then centrifuged, resuspended, and subjected to a second and a third round of immunoselection by adding fresh Dynabeads and incubating another 90 min for each round. Finally, cells that were not firmly bound to Dynabeads during three rounds of selection were resuspended in PBSF, plated on YPD plates, and incubated for 3–4 d at 24°C. YPD plates were replicated, and replica plates were incubated for 2 d at 37°C to allow for selection of clones that are temperature sensitive (ts) for growth. ts mutants were tested for acid phosphatase secretion by replica plating clones grown at 24°C onto phosphate-free SDCUA plates, and incubating the replicas at 37°C for 1–2 d. Replica plates were then processed for the detection of secreted phosphatase as described (21). Mutants able to secrete acid phosphatase at 37°C were picked to be further screened for GPI defects.

### Radiolabeling with [2-<sup>3</sup>H]myo-inositol

For further analysis, mutant strains were labeled with [2-<sup>3</sup>H]myo-inositol ([2-<sup>3</sup>H]Ins). Cells were grown in SDCUA medium, 2.5 OD<sub>600</sub> U of exponentially growing cells were resuspended in 0.25 ml SDCUA, preincubated 20 min at 37°C, and 5  $\mu$ Ci [2-<sup>3</sup>H]Ins were added. Cells were then incubated for 20 min at 37°C, and diluted with 1 ml of prewarmed SDCUA, and further incubated for 70 min at 37°C. At the end, cells were washed with water. Lipids were extracted and desalted by butanol extraction as described (70). Lipid extracts were analyzed by ascending TLC using 0.2-mm-thick silica gel plates with the solvent system chloroform/methanol/water (10:10:3). As standards, we used previously characterized [2-<sup>3</sup>H]Ins- and [2-<sup>3</sup>H]mannose-labeled lipids from *S. cerevisiae*. (61, 70). Radioactivity was detected by two-dimensional radioscanning (LB 2842; Berthold AG, Regensdorf, Switzerland) or else, TLC plates were sprayed with EN<sup>3</sup>HANCE and exposed to film (X-OMAT; Eastman Kodak Co., Rochester, NY) at –80°C.

For protein analysis, Laemmli sample buffer (32) was added to labeled cells (25  $\mu$ l/OD<sub>600</sub>), and cells were broken by vortexing with glass beads (3  $\times$  1 min). Samples were boiled 5 min at 95°C, centrifuged 10 min at 10,000 g to remove cell walls, and the supernatant was run on an SDS-PAGE gradient gel 7.5–15% (32). Gels were treated with EN<sup>3</sup>HANCE and exposed to Kodak X-OMAT film at –80°C.

### Analysis of Abnormal [2-<sup>3</sup>H]myo-inositol-labeled Lipids

Purification of radiolabeled glycolipids was achieved by preparative TLC in the same solvent system as above, radioactive glycolipids of interest were localized by two-dimensional radioscanning, and were scraped and eluted with the solvent. Before further analysis, an aliquot of purified glycolipids was run on analytical TLC to check for purity.

Soluble head groups were obtained from purified glycolipids through selective removal of acyl groups attached to the Ins using methanolic NH<sub>3</sub>, followed by PI-PLC treatment in 20% 1-propanol (60). Fatty acids and lipids were removed by butanol extraction. Head groups were subjected to hydrofluoric acid (HF) dephosphorylation and then to N-acetylation, and were desalted using mixed-bed ion exchange resin AG-501-X8 (Bio-Rad Laboratories, Hercules, CA) (60). N-acetylated, water-soluble head

group fragments were separated by horizontal silica gel 60 HPTLC in three consecutive runs in solvent A (1-propanol/acetone/water 9:6:5, vol/vol) and solvent B (1-propanol/acetone/water 5:4:1, vol/vol) used in the order A, B, A as described (68). Previously described methods were used for mild base hydrolysis (18), jack bean  $\alpha$ -mannosidase (JBAM) treatment (62), and GPI-PLD treatment (12) of lipid mixtures. Treated lipid mixtures were desalted by extraction with butanol before TLC.

### Measurement of Invertase Secretion

To measure periplasmic invertase in intact cells, the cells were grown to mid-logarithmic phase in SDCUA with the glucose content raised to 5% (SD<sub>5</sub>CUA) at 24°C with shaking. 10 OD<sub>600</sub> U of cells were pelleted, resuspended in 20 ml SD<sub>5</sub>CUA, and preincubated for 60 min at 37°C. Cells were then pelleted, washed with water at 37°C, resuspended in 20 ml of SDCUA with the glucose content reduced to 0.05% (SD<sub>0.05</sub>CUA), and incubated at 37°C. 200- $\mu$ l aliquots were removed every 15 min during 1 h. These aliquots were adjusted to 10 mM NaN<sub>3</sub>, 10 mM NaF, and 10  $\mu$ g/ml cycloheximide, and were kept on ice until they were washed three times with water and external invertase activity was measured (19). The OD<sub>600</sub> of each aliquot was measured to allow calculation of the invertase activity per OD<sub>600</sub> U of cells.

### Invertase Glycosylation

Cells were grown to mid-logarithmic phase in SD<sub>5</sub>CUA at 24°C with shaking. 5-OD<sub>600</sub> U aliquots were resuspended in 10 ml SD<sub>5</sub>CUA, and incubated for 60 min at 24°C or 37°C, respectively. Cells were then washed with water at 24°C or 37°C, and resuspended in SD<sub>0.05</sub>CUA, and incubated for 60 min at 24°C or 37°C, respectively. Metabolism was then arrested by the addition of 10 mM NaN<sub>3</sub>, 10 mM NaF, and 10  $\mu$ g/ml cycloheximide, and extracts prepared for native gel electrophoresis and activity stain as described (2).

### Expression of $\alpha$ -agglutinin at the Cell Surface

Cells were grown at 24°C to an OD<sub>600</sub> of 1–2 in SDCUA, and buffered to pH 5.0. Cells were resuspended in fresh SDCUA, pH 5, and 0.16-ml aliquots (0.625 OD<sub>600</sub>/ml) of cells were either stimulated by a-factor (70 U/ml, final concentration), or control incubated for 3 h at 24°C or 37°C, respectively. Cells were then adjusted to 10  $\mu$ g/ml cycloheximide, washed with 1 ml buffer A (10 mM Tris/HCl, pH 7.4, 100 mM MgCl<sub>2</sub>, 0.5% Tween 20, 1% Triton X-100, 1% BSA, 5% FCS, 10  $\mu$ g/ml cycloheximide). Cells were resuspended in 90  $\mu$ l buffer A, 10  $\mu$ l rabbit anti- $\alpha$ -agglutinin antiserum was added, and incubation was continued for 90 min at 24°C. Cells were washed three times with 1 ml buffer A, and resuspended in 250  $\mu$ l buffer A. 1  $\mu$ l anti-rabbit-IgG/alkaline phosphatase conjugate was added, and cells were incubated for 45 min at 24°C. Cells were washed five times with buffer A, and alkaline-phosphatase activity was measured by incubation of cells with paranitrophenylphosphate during 10 min as described (79). No activity was observed when the second antibody was omitted.

### Maturation of Gas1p

Maturation of Gas1p was followed by pulse-chase experiments using [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (Tran35/S label; ICN Biomedicals Inc., Irvine, CA), 1,027 Ci/mmol; 20  $\mu$ Ci/OD<sub>600</sub> of cells) followed by immunoprecipitation using a polyclonal rabbit anti-Gas1p antiserum (17) and protein A-Sepharose as described (26). Western blotting was carried out with the same antiserum as described (26).

## Results

### Elaboration of Conditions for Immunoselection

Mutants with defects in the structural genes of a- and  $\alpha$ -agglutinins previously have been obtained by selecting for cells unable to form aggregates when mixed with cells of the opposite mating type (34, 65). This approach was relatively unsuccessful for obtaining GPI-deficient mutants, possibly because this procedure selects for a complete absence of agglutinins from the surface, so that cells exhibiting constitutive levels of agglutinins are eliminated,

and only cells with defects in the agglutinin structural genes are selected. Thus, a less restrictive immunoselection procedure using a highly specific polyclonal rabbit antibody against  $\alpha$ -agglutinin was developed. As can be seen in Fig. 1, W303-1B MAT $\alpha$  cells, when induced with a-factor, were strongly stained by the antiserum, whereas only vacuolar staining was visible in noninduced cells. In preliminary experiments, W303-1B MAT $\alpha$  cells were first induced with a-factor at 37°C, and were then incubated with anti- $\alpha$ -agglutinin antibodies. Absorption of these cells onto anti-IgG-coated magnetic beads was very efficient, even if cells had not been induced to express  $\alpha$ -agglutinin before. However, after extensive washing of the beads, only 4% of noninduced, but 85% of induced cells remained fixed to the beads. Thus, through three consecutive rounds of adsorption onto magnetic beads, followed each time by extensive washings, cells not expressing  $\alpha$ -agglutinin at 37°C could theoretically become enriched up to 300-fold.

### Immunoselection of GPI Mutants

Mutants were selected using the procedure outlined in Fig. 2. Strain W303-1B was mutagenized with ethyl methanesulfonate, and surviving cells (21%) were allowed to recover in SDCUA through six mitotic cell cycles at 24°C. 10<sup>8</sup> mutagenized cells were then induced to express  $\alpha$ -agglutinin at 37°C, and the cells expressing more than constitutive levels of  $\alpha$ -agglutinin were removed using three consecutive rounds of immunoselection. The negatively selected remaining cells (8.7%) were assumed to comprise all the dead cells generated by mutagenesis. On the basis of this assumption, we calculate that the immunoselection had removed 97% of living cells. The negatively selected cells were then plated on YPD plates for growth at 24°C. The

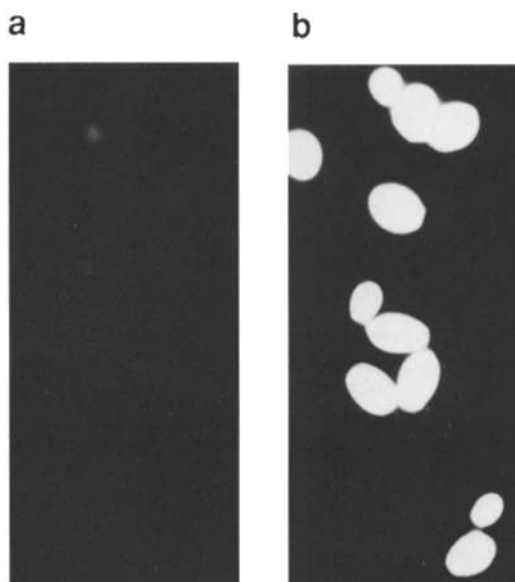


Figure 1.  $\alpha$ -Agglutinin expression by  $\alpha$  cells as detected by anti- $\alpha$ -agglutinin antibodies. W303-1B cells were incubated for 90 min in the absence (a) or presence (b) of a-factor, and decorated with polyclonal anti- $\alpha$ -agglutinin antibodies followed by FITC-conjugated goat anti-rabbit-IgG antibodies. Light microscopic pictures were taken using the same exposure times for a and b.

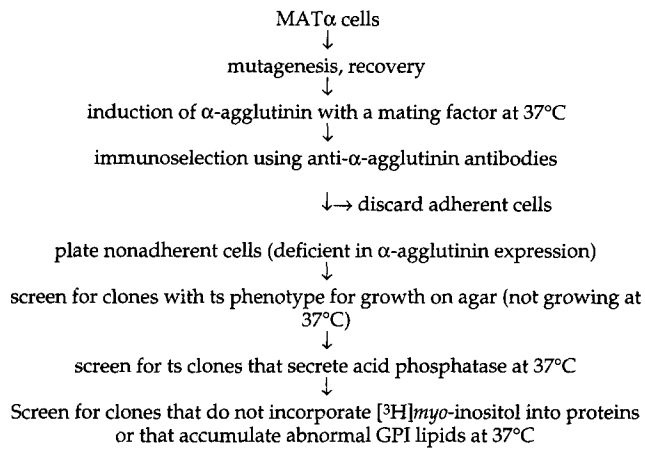


Figure 2. Protocol for selection of *gpi* mutants.

plating efficiency was only 10%, suggesting that a major fraction of the selected cells indeed were dead cells left over from the mutagenesis. Of the growing clones, those that did not grow at 37°C were selected (20%), and these *ts* clones were further screened for acid phosphatase secretion at 37°C. 72% of *ts* clones secreted acid phosphatase normally, and these clones were labeled with [2-<sup>3</sup>H]Ins. Lipid extraction, TLC, and fluorography were used to screen for the presence of abnormal [2-<sup>3</sup>H]Ins-labeled lipids, whereas the incorporation of [2-<sup>3</sup>H]Ins into proteins was monitored by SDS-PAGE and fluorography. 10 mutant isolates accumulated a distinct, abnormal [2-<sup>3</sup>H]Ins-labeled lipid, and one mutant (*gpi9*) accumulated one major and several minor abnormal species (Fig. 3). In all 11 mutants, the amount of [2-<sup>3</sup>H]Ins-labeled proteins accumulated during a 90-min pulse was significantly reduced as compared with wild type. This reduction was observed in all mutants when cells were labeled at 37°C, and in many mutants even after labeling at 24°C (Fig. 4). On the other

hand, all mutants incorporated [2-<sup>3</sup>H]Ins into phospholipids as efficiently as wild-type cells (50–80% of added radioactivity). In none of 400 other isolates was the incorporation of [2-<sup>3</sup>H]Ins into proteins abolished. Restesting of the growth phenotype showed that several of the 11 isolates that initially had scored as *ts* were in fact not temperature sensitive for growth (Table I). In most mutants, the accumulation of the abnormal lipid was enhanced when cells were labeled at 37°C, but in several mutants, the abnormal lipid could also be observed after labeling at 24°C (Fig. 3, Table I). The 11 mutant isolates were outcrossed three times to X2180, and all tetrads were labeled with [2-<sup>3</sup>H]Ins at 37°C. The accumulation of an abnormal lipid segregated 2:2 in all cases and the *ts* growth phenotype, if present, cosegregated with the abnormal lipid in all but one isolate (*gpi7-1*). Also, the accumulation of distinct abnormal lipids behaved as a stable trait, and the same lipids as in the original isolate were consistently observed in segregants. *gpi7-4* and *gpi8* stem from a single mutant isolate containing two independent GPI defects; during crossing of this isolate, two different spore phenotypes accumulating two different abnormal lipids were obtained. The two phenotypes were unlinked, and consistently segregated 2:2 through three further outcrosses. One mutant (isolate 839) could not be mated. For complementation analysis, all mutants accumulating the same abnormal lipid were crossed with each other and also with the isolate that accumulates a range of different abnormal lipids (*gpi9*, Fig. 3). Diploids were checked for the accumulation of abnormal lipids and, if *ts* strains were crossed, for growth at 37°C. The five isolates that accumulated lipid  $\alpha$  were also crossed with *sec53*, *sec59*, *dpml1*, and *pml40*, a series of *ts* mutants with known defects in mannose biosynthesis or mannose utilization. These mutants also accumulate lipid  $\alpha$ . All five isolates, and all four known mutants accumulating lipid  $\alpha$  complemented each other, except for one of our isolates that did not complement *sec53*. This isolate was not studied further. Our isolates accumulating lipid  $\alpha$  were classified as

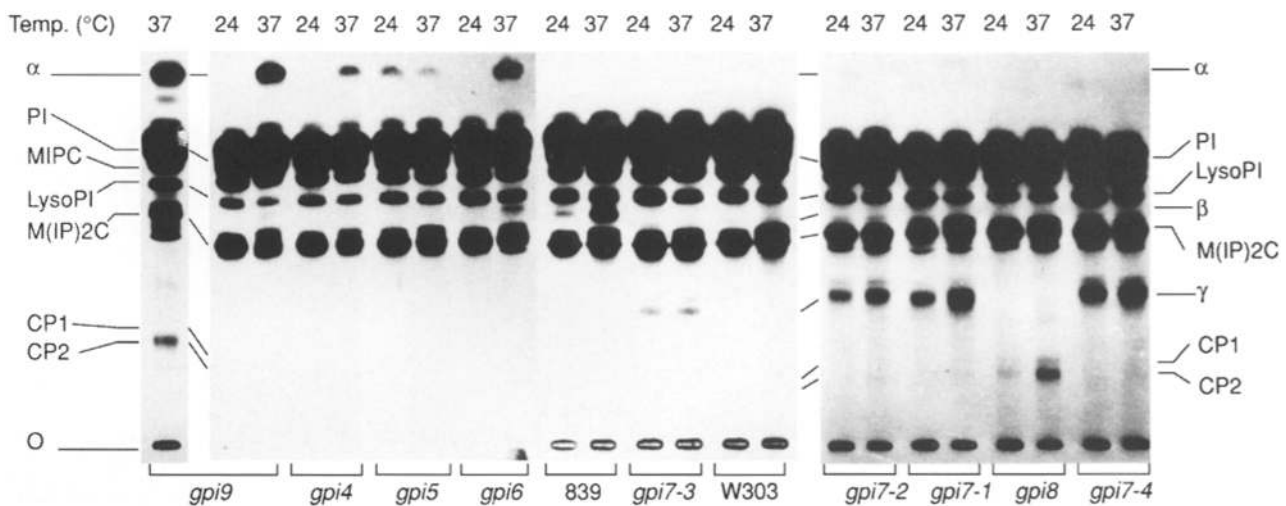
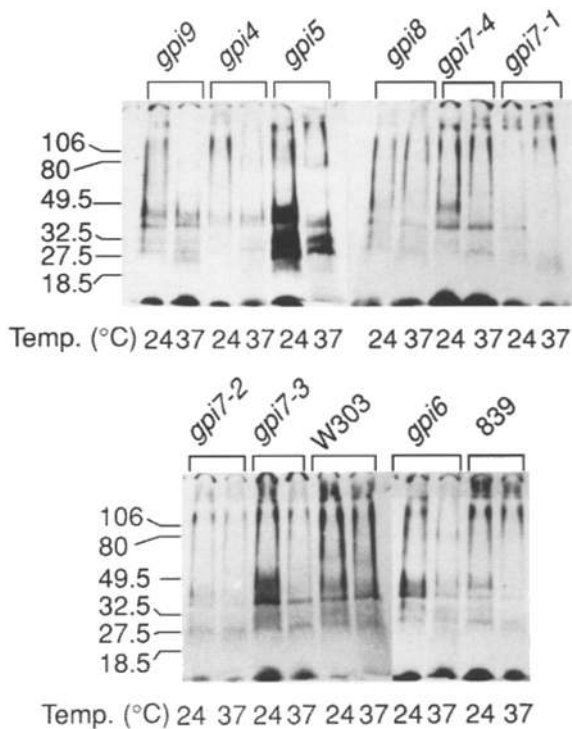


Figure 3. Accumulation of abnormal lipids in *gpi* mutants. Lipids from mutant cells labeled at 24°C or 37°C with [2-<sup>3</sup>H]Ins were run on TLC and revealed by fluorography for 6 d. The first lane on left represents an experiment where a threefold longer exposure had been performed allowing a better view of the minor CP of *gpi9*. As will be shown below, lipid  $\alpha$  corresponds to GlcN-acylIns-P-lipid;  $\beta$  to Man<sub>2</sub>-GlcN-acylIns-P-lipid;  $\gamma$  to Man<sub>4</sub>-GlcN-acylIns-P-lipid. O, origin.



**Figure 4.** Incorporation of [ $2\text{-}^3\text{H}$ ]Ins into proteins of *gpi* mutants.  $3 \times$  outcrossed mutant strains were grown to mid-log phase, and were labeled with [ $2\text{-}^3\text{H}$ ]Ins. Proteins were extracted, run in SDS-PAGE, and revealed by fluorography. Each lane contains the total extract of  $2.5 \text{ OD}_{600} \text{ U}$  of cells labeled with  $5 \mu\text{Ci}$  of [ $2\text{-}^3\text{H}$ ]Ins. It should be noted that mature GPI-anchored proteins are extensively and heterogeneously glycosylated, which leads to crowding at the border of the lane and smearing out (33, 55). In parallel, the four strains from individual tetrads of the third outcross (see below) of each mutant were labeled with [ $2\text{-}^3\text{H}$ ]Ins to determine the efficiency of incorporation of [ $2\text{-}^3\text{H}$ ]Ins into mutant strains. Efficiency of incorporation was calculated by dividing the mean of cpm in the lipid extracts of the two mutants by the mean of cpm of the two wild-type strains derived from a single tetrad. Given as percentage of wild type, mutants incorporated with efficiencies of 104% (*gpi4*), 72% (*gpi5*), 81% (*gpi6*), 95% (*gpi7-1*), 123% (*gpi7-2*), 108% (*gpi7-3*), 120% (*gpi7-4*), 155% (*gpi8*), 124% (*gpi9*).

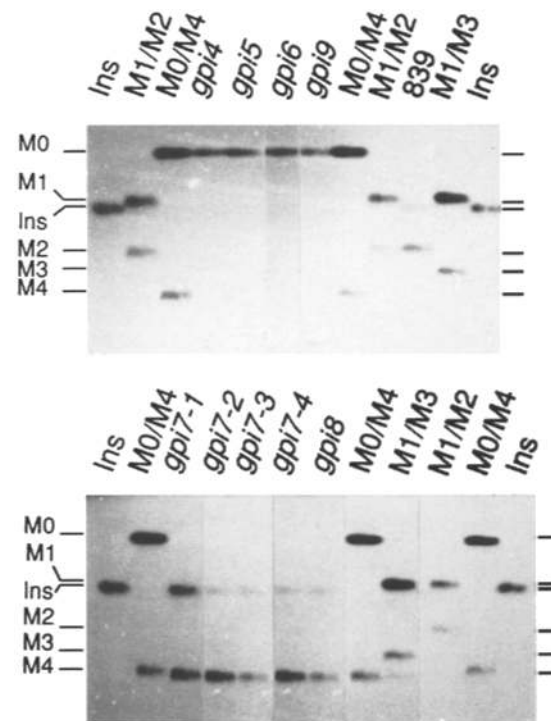
*gpi4*, *gpi5*, *gpi6*, and *gpi9*. Four isolates accumulating lipid  $\gamma$  did not complement each other, and were classified as *gpi7*. Labeling of diploid strains also allowed to conclude that lipid accumulation in *gpi4*, *gpi5*, *gpi6*, *gpi7*, *gpi8*, and *gpi9* is a recessive trait.

### Characterization of Abnormal Lipids

Structural analysis was performed on the abnormal lipids of mutants to see whether or not they represent biosynthetic intermediates of the GPI pathway. For this, [ $2\text{-}^3\text{H}$ ]Ins-labeled abnormal lipids were isolated from preparative TLC plates, and were fragmented using HF. Under the conditions used, HF specifically cleaves phosphoester bonds, and generates  $\text{Man}_x\text{-GlcN-Ins}$  ( $x = 0, 1, 2, 3, 4$ ) from GPI biosynthetic intermediates. *N*-acetylated HF fragments of the abnormal lipid  $\alpha$  isolated from *gpi4*, *gpi5*, and *gpi6* comigrated with GlcNAc-Ins, the fragment of

lipid  $\beta$  from mutant 839 with  $\text{Man}_2\text{-GlcNAc-Ins}$  (Fig. 5). Fragments of lipid  $\gamma$  from *gpi7* and of CP1/CP2 from *gpi8* comigrated with  $\text{Man}_4\text{-GlcNAc-Ins}$  (Fig. 5). Thus, HF fragments of the major abnormal lipid of *gpi7* and *gpi8* are identical, but only the lipid of *gpi8* comigrates with CP's on TLC, whereas lipid  $\gamma$  of *gpi7* is less polar. It therefore is possible that the lipid of *gpi7* lacks the polar phosphoethanolamine substituent characteristic of CP's. Lipid  $\alpha$  of *gpi9* produced a GlcNAc-Ins fragment, but this mutant contains several additional lipids the most polar of which comigrates with CP2 (Fig. 3).

The three GPI biosynthesis intermediates of yeast characterized so far contained an acyl chain on the Ins, rendering them resistant to PI-PLC (9, 55, 70). The [ $2\text{-}^3\text{H}$ ]Ins-labeled lipid extracts of all mutants were treated with PI-PLC and analyzed by TLC/fluorography. All abnormal mutant lipids were resistant to PI-PLC, whereas PI was completely hydrolyzed as expected (data not shown). Thus, by their comigration with well-characterized GPI intermediates on TLC, by the comigration of their HF fragments with GPI-derived HF fragments, and by their resistance to PI-PLC, the abnormal [ $2\text{-}^3\text{H}$ ]Ins-labeled mutant lipids behave as biosynthetic intermediates of the GPI pathway. To con-



**Figure 5.** Thin layer chromatogram of HF fragments generated from mutant lipids. The major abnormal [ $2\text{-}^3\text{H}$ ]Ins-labeled lipid of each mutant was isolated by preparative TLC, HF fragments were prepared and compared with known standard oligosaccharides by TLC. The lipids analyzed were lipid  $\alpha$  (*gpi4*, *gpi5*, *gpi6*, *gpi9*),  $\beta$  (mutant 839),  $\gamma$  (*gpi7*), and CP1/CP2 (*gpi8*), the positions of which can be seen in Fig. 3. M0–M4,  $\text{Man}_0$ – to  $\text{Man}_4$ -GlcNAc-Ins (70). Mixtures of two of these standards and Ins were run in parallel on the same plate, and the positions of the respective standards were highlighted on the borders. The presence of Ins in 839 and *gpi7* is probably due to unadverted hydrolysis by HF favored by heat production during neutralization at the end of the HF treatment.

firm that the abnormal lipid accumulating in *gpi8* is identical with CP1/CP2 (Fig. 3), we performed additional chemical or enzymatic treatments. Thereby the abnormal lipid of *gpi8* was compared with the well-characterized CP's contained in the [2-<sup>3</sup>H]mannose-labeled lipid extract of *pmi40*, a mutant which, at 37°C, incorporates high amounts of [2-<sup>3</sup>H]mannose due to a temperature-dependent defect of mannose biosynthesis (57, 70). As reported previously, all lipid moieties of CP1 and CP2 are removed by mild base hydrolysis; they are cleaved by GPI-PLD and only three of the four mannoses of the isolated head groups are protected against JBAM (70). As shown in Fig. 6, treatment of CP1 and CP2 with JBAM increases their mobility to the extent that is expected for the removal of a single mannose residue. The abnormal lipid of *gpi8* shows the same behavior as the authentic CP2 from *pmi40* in all treatments. We thus conclude that *gpi8* accumulates a complete GPI precursor. To confirm that lipid  $\gamma$  of *gpi7* is an earlier intermediate of GPI biosynthesis, the double mutant *gpi7-1 gpi8* was constructed. The double mutant only accumulated lipid  $\gamma$  (not shown). Thus, *gpi7* is epistatic to *gpi8*, a finding consistent with the idea that lipid  $\gamma$  is a precursor of CP2.

### Secretion of Invertase Is Independent of the Surface Expression of $\alpha$ -agglutinin and Maturation of Gas1p

While mutants deficient in the surface expression of  $\alpha$ -agglutinin but able to secrete acid phosphatase had been selected, the phenotype of these mutants with regard to secretion of

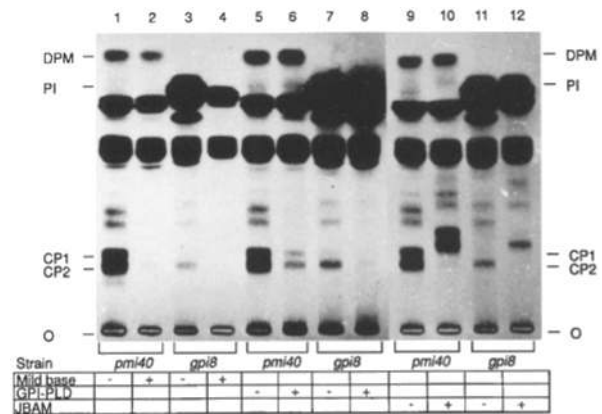


Figure 6. Characterization of the abnormal lipid accumulating in *gpi8*. *Gpi8* was labeled with [2-<sup>3</sup>H]Ins, *pmi40* with [2-<sup>3</sup>H]-mannose (70), both labelings at 37°C. Desalted lipid extracts were subjected to mild base hydrolysis, GPI-PLD treatment, or JBAM treatment as indicated at the bottom (+). Control incubations without the hydrolytic agent were done in parallel (-). Treated lipids were desalted and analyzed by TLC/fluorography. DPM, dolicholphosphomannose.

soluble proteins and surface expression of  $\alpha$ -agglutinin had to be verified. Invertase was induced by glucose deprivation both after a 10-min and a 1-h preincubation at 37°C. The latter time was chosen to demonstrate invertase secretion in the time window during which  $\alpha$ -agglutinin normally appears at the cell surface (36). As can be seen in

Table I. Summary Table

Complementation class	°C	<i>gpi4-1</i>	<i>gpi5-1</i>	<i>gpi6-1</i>	839	<i>gpi7-1</i>	<i>gpi7-2</i>	<i>gpi7-3</i>	<i>gpi7-4</i>	<i>gpi8-1</i>	<i>gpi9-1</i>	W303
Growth	24	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++
	37	-	+++	-	+	++	+++	+++	++	++	-	+++
Accumulation of abnormal lipid (Fig. 3)	24	-	+	-	+	+	+	+	++	-	-	-
	37	+	+	+++	+++	+++	++	+	+++	+	+++	-
Growth on 1 mg/ml CFW (Table I)	24	++	+++	+++	+	+++	+	+++	+++	++	+++	+++
	37	-	-	-	-	-	-	++	-	-	-	+++
Secretion of invertase (Fig. 7)	37	+++	++	+	+++	+++	+++	++	+++	+++	+++	+++
Induction of $\alpha$ -agglutinin on cell surface at 37°C (Fig. 8)	37	-	+	-	-	+++	-	+	+++	+++	-	+++
Accumulation of immature Gas1p (Fig. 9)	24	n.t.*	n.t.	n.t.	n.t.	+	++	++	++	+	n.t.	+
	37					+	+	++	++	++		+
Slowed export of Gas1p out of ER (Fig. 10)	37	n.t.	n.t.	n.t.	++	+	n.t.	n.t.	n.t.	++	n.t.	-
Hypersensitivity to vanadate	24	-	-	-	-	-	-	-	-	-	-	-
	37	n.t.	-	n.t.	+	-	-	-	+	+	n.t.	-
Sensitivity to hygromycin B (50 $\mu$ g/ml)	24	-	-	-	-	-	-	-	+	-	-	-
	37	n.t.	+	n.t.	++	+	-	+	++	-	n.t.	-
Growth on Ca <sup>++</sup> (50 mM)	24	no. <sup>‡</sup>	no.	no.	no.	no.	no.	no.	no.	no.	no.	no.
	37	de. <sup>§</sup>	rd. <sup>  </sup>	no.	rd.	no.	no.	no.	no.	no.	no.	no.
Status of N-glycans of invertase (Fig. 11)	24	no.	no.	no.	no.	no.	no.	no.	no.	no.	no.	no.
	37	s.r. <sup>¶</sup>	no.	s.r.	no.	no.	no.	no.	no.	no.	rd.	no.
Incorporation of [2- <sup>3</sup> H]Ins into proteins	24	+	+++	++	++	+	+	+++	++	+	++	+++
	37	(+)	++	+	+	+	+	+	++	+	+	+++
Size of HF fragment of abnormal GPI lipid	37	M0	M0	M0	M2	M4	M4	M4	M4	CP1 CP2	**M0 CP2	-

\*. Not tested;

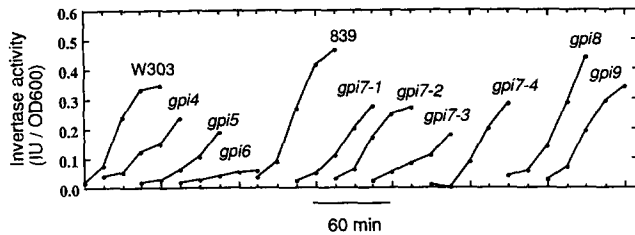
‡, normal;

§, dependent;

||, reduced;

¶, slightly reduced;

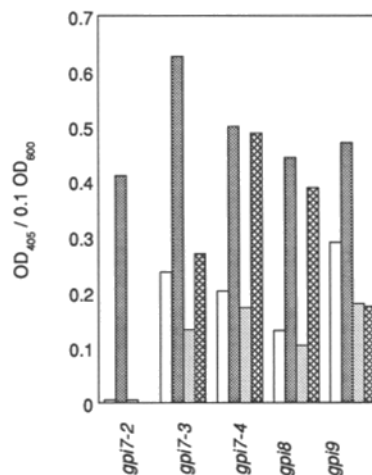
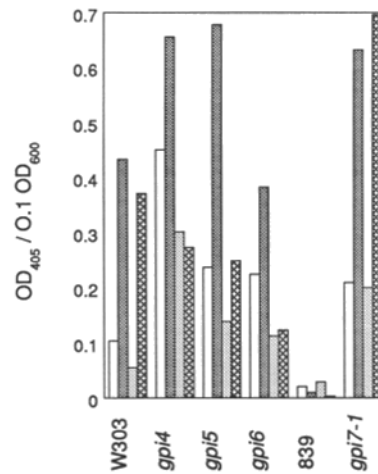
\*\*, accumulates several intermediates from GlcN-acylPI up to CP2.



**Figure 7.** Secretion of invertase by *gpi* mutants.  $3 \times$  outcrossed strains were grown to mid-log phase at 24°C, were preincubated in SD<sub>5</sub>CUA for 1 h at 37°C, and invertase secretion was induced by transferring cells into SD<sub>0.05</sub>CUA. After further incubation at 37°C for 0, 15, 30, 45, or 60 min, periplasmic invertase activity was determined.

Fig. 7, invertase was secreted into the periplasmic space by all mutants except for *gpi6*, which secreted normally immediately upon shift to 37°C (not shown), but was unable to secrete invertase after 1 h of preincubation at 37°C. Incidentally, normal secretion of invertase also documents that a given mutant has no defects in transcription (4, 5), translation, and translocation of secretory proteins.  $\alpha$ -Agglutinin expression was measured by an ELISA test, whereby the conditions and the duration of induction with a factor were the same as in the initial mutant selection procedure (Fig. 8). A conceptual difficulty of this assay lies in the fact that  $\alpha$ -agglutinin expression was tested after three outcrosses to X2180, a strain which has a relatively high, constitutive level of expression, and which induces  $\alpha$ -agglutinin only 1.3-fold (79). Thus, the different mutants are not isogenic and cannot be compared among each other, but each mutant has to be considered independently. In doing so, one can see that in all but three mutants, the induction of  $\alpha$ -agglutinin expression on the cell surface at 37°C is significantly lower than at 24°C. Exceptions are *gpi7-1*, *gpi7-4*, and *gpi8*. These mutants can be assumed to have filtered through the original immunoselection, because *gpi7-4* and *gpi8* were present in the same clone, and *gpi7-1* was combined with another, unrelated mutation causing a *ts* growth phenotype (see above). Thus, it is conceivable that the combination of mutations in these original isolates resulted in a low expression of  $\alpha$ -agglutinin at 37°C.

To test further whether GPI anchor addition was compromised, the biosynthesis of Gas1p was studied in the mutants blocked at later stages of GPI biosynthesis. On SDS-PAGE, the ER form of Gas1p has an apparent molecular mass of 105 kD; upon entry into the Golgi apparatus, the elongation of *N*- and *O*-glycans increases the size to 125 kD (17, 74). Gas1p is retained in the ER as long as the GPI anchor is not attached to it (26, 53). Protein extracts from mutants precultured at 24° or 37°C were analyzed by Western blotting with anti-Gas1p antibody. As can be seen in Fig. 9, as compared to wild-type cells, the relative amount of the ER precursor form of Gas1p was increased in several mutants already when they were grown at 24°C, and this tendency was enhanced in cells grown at 37°C. This suggested that the maturation of Gas1p was delayed in these mutants. To further document this idea, the transit of Gas1p from the ER to the Golgi apparatus was studied by pulse-chase experiments in *gpi7-1*, *gpi8*, and 839, three strains which secrete invertase nor-

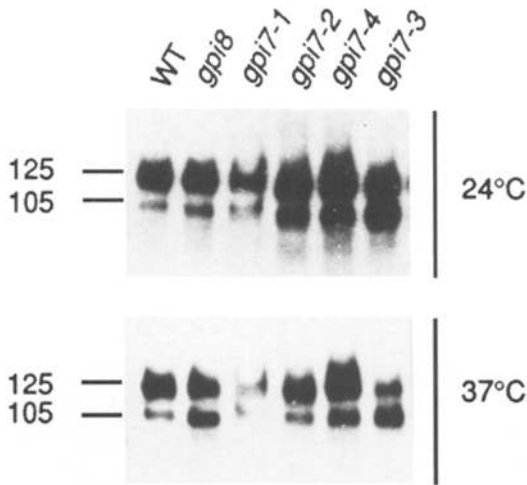


**Figure 8.** Surface expression of  $\alpha$ -agglutinin by *gpi* mutants.  $\alpha$ -Agglutinin was induced with a-factor at 24°C or 37°C during 3 h, and the amount of  $\alpha$ -agglutinin at the surface was then quantitated by ELISA. Results are given as the absorbance at 405 nm produced by reagents bound to 0.1 OD<sub>600</sub> U of cells in 10 min at 37°C.  $3 \times$  outcrossed mutants were analyzed. □, 24°C non-induced; ■, 24°C induced; ▒, 37°C non induced; ⊠, 37°C induced.

mally (Fig. 7). As shown in Fig. 10, maturation of the pulse-labeled 105-kD ER form of Gas1p into the mature 125-kD form during chase at 37°C was severely delayed in *gpi8* and 839, and somewhat delayed in *gpi7-1*, but eventually still occurred. The delay can account for the accumulation of immature Gas1p observed by Western blotting (Fig. 9); the fact that maturation of Gas1p is eventually still possible is in keeping with the fact that these strains still can grow at 37°C.

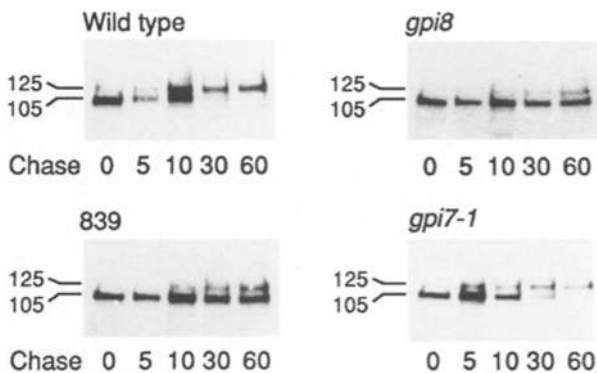
#### *N*-Glycosylation of Most *gpi* Mutants Is Normal

Mutants deficient in the biosynthesis of the dolichol-linked oligosaccharide precursors for *N*-glycosylation (*alg2*, *alg3*) are not deficient in the biosynthesis of GPI anchors (8), nor do they accumulate any abnormal [<sup>3</sup>H]Ins-labeled lipids (data not shown). Nevertheless, we wanted to rule

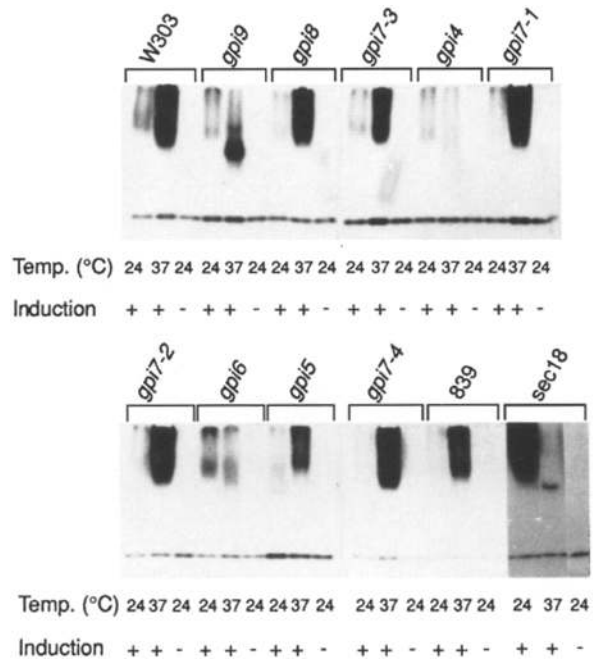


**Figure 9.** *Gpi7* and *gpi8* contain increased amounts of immature Gas1p. 3 × outcrossed strains were grown to mid-log phase at 24°C, and were then further incubated for 1 h at 24°C or 37°C. Proteins were extracted, separated in SDS-PAGE, and blotted. Western blotting was carried out with rabbit anti-Gas1p followed by sheep anti-rabbit-IgG/peroxidase, and bands were revealed by chemiluminescence.

out that the accumulation of abnormal GPI intermediates in *gpi* mutants was an indirect consequence of more general perturbations of the ER and the secretion pathway. Since perturbations of this kind very often compromise the N-glycosylation and/or the elongation of N-glycans, we chose to monitor the N-glycosylation of invertase. The electrophoretic mobility of secretory invertase in native gels is a good indicator of the status of N-glycans on this glycoprotein (1, 2). Native gel electrophoresis showed that invertase of *gpi9* is underglycosylated to the same extent as the invertase retained in the ER of *sec18* cells, that invertase of *gpi4* and *gpi6* is partially underglycosylated, and that the other *gpi* mutants glycosylated invertase normally (Fig. 11). This suggests that *gpi4*, *gpi6*, and *gpi9* contain a pleiotropic mutation affecting the GPI biosynthesis pathway, as well as N-glycosylation. It should be noted that



**Figure 10.** Kinetics of export of Gas1p out of the ER. W303 cells or 3 × outcrossed mutant cells were grown to mid-log phase, were preincubated for 60 min at 37°C, and labeled for 10 min with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, and then chased for 0, 5, 10, 30, or 60 min with cold Met and Cys at 37°C as indicated. Gas1p was immunoprecipitated and analyzed by SDS-PAGE.



**Figure 11.** N-glycosylation of invertase by *gpi* mutants. 3 × outcrossed mutant strains were grown to mid-log phase, and were preincubated at 24°C or 37°C for 60 min. Invertase secretion was induced (+) by a further 60 min incubation in glucose-deprived medium at the same temperatures. Alternatively, cells were control incubated (-) in medium containing 5% glucose at 24°C. Cells were broken, the cell sap was subjected to electrophoresis in a nondenaturing gel system, and invertase was detected by addition of a chromogenic substrate.

these mutants accumulate the same GPI intermediate (GlcN-acylPI) as *pmi40*, *sec53*, *sec59*, and *dpm1*, all mutants deficient in the biosynthesis or activation of mannose (9, 55, 70). The accumulation of the abnormal lipid of *gpi4*, *gpi5*, *gpi6*, and *gpi9* mutants persists even if mannose is added to the culture medium (not shown). Thus, *gpi4*, *gpi6*, and *gpi9* may be further genes required for the activation of mannose.

### ***Gpi* Mutants Are Hypersensitive to Calcofluor White and Hygromycin B**

Sequence analysis of several cell wall proteins reveals the presence of COOH-terminal hydrophobic sequences that may direct the attachment of a GPI anchor (72), and for certain proteins, the GPI anchor represents a necessary and sufficient signal allowing them to get targeted and integrated into the cell wall (69, 80). These findings led us to probe the intactness of the cell walls of *gpi* mutants with calcofluor white (CFW) (64). As shown in Table II, wild-type cells are not inhibited by 1.5 mg/ml of CFW, whereas all mutants that are not ts for growth in the absence of CFW are inhibited by the dye at 37°C. On the other hand, five mutants (*gpi4*, *gpi7-2*, *gpi7-4*, *gpi8*, and *gpi9*) are not able to grow in high concentrations of CFW already at 24°C, indicating that their defect can be made apparent already at 24°C.

An increased fragility of the cell wall at 37°C was also indicated by the fact that several mutants did not grow in the



Table II. Calcofluor White Sensitivity of Yeast GPI Mutants

Strain		CFW concentration mg/ml			
		0	0.5	1	1.5
W303	24°C	+++	+++	+++	+++
	37°C	+++	+++	+++	+++
<i>gpi4-1</i>	24°C	+++	+++	++	-
	37°C	-	-	-	-
<i>gpi5-1</i>	24°C	+++	+++	+++	+++
	37°C	++	+	-	++
<i>gpi6-1</i>	24°C	+++	+++	+++	+++
	37°C	-	-	-	-
839	24°C	+++	+	+	-
	37°C	++	-	-	-
<i>gpi7-1</i>	24°C	+++	+++	+++	+++
	37°C	++	+	-	-
<i>gpi7-2</i>	24°C	+++	+++	+	+
	37°C	++	-	-	-
<i>gpi7-3</i>	24°C	+++	+++	+++	+++
	37°C	+++	+++	++	+
<i>gpi7-4</i>	24°C	+++	+++	+++	++
	37°C	++	+	-	-
<i>gpi8-1</i>	24°C	+++	++	++	-
	37°C	+	-	-	-
<i>gpi9-1</i>	24°C	+++	+++	+++	++
	37°C	-	-	-	-

Mutant cells were plated out on YPD agar plates containing 0, 0.5, 1, or 1.5 mg/ml of CFW. Growth at 24° or 37°C was monitored after 2 d of incubation. Size of emerging clones was rated +++, normal; ++, small; +, very small; and -, no colonies.

presence of 50 or 100  $\mu$ M hygromycin at 37°C, whereas most mutants grew normally in hygromycin at 24°C (Table II) (3).

*Mnn9*, a mutant deficient in *N*-glycan elongation, has a fragile cell wall (1), is hypersensitive to CFW (64), and has also been reported to have an increased resistance to vanadate (3). Therefore, we wanted to verify the vanadate resistance of *gpi* mutants. W303 and X2180 cells were resistant to vanadate up to a concentration of 5 mM. None of the mutants had a higher resistance if tested at 24°C or 37°C (Table I). To the contrary, several mutants exhibited a reduced colony size when plated at 3 or 5 mM vanadate. In three strains, growth at 37°C was influenced by the  $Ca^{++}$  content of the medium. *Gpi5* and 839 almost ceased to grow at 50 mM  $Ca^{++}$ , whereas *gpi4* was  $Ca^{++}$ -requiring, i.e., it grew to normally sized colonies at 37°C if plated on 50 mM  $Ca^{++}$ .

## Discussion

We have developed a new approach for the selection of GPI-anchoring mutants in yeast. The procedure selects cells that do not express a GPI protein at the cell surface, and follows the same strategy as was used to isolate mammalian GPI-anchoring mutants (28). Since GPI anchoring has been claimed to be a vital process for yeast cells, it was necessary to select for temperature-sensitive mutants.  $\alpha$ -Agglutinin, although minor among the numerous GPI-anchored proteins, represents an ideally suited target antigen for immunoselection. It can be induced after a temperature shift, and it becomes detectable on the outside of the cell wall, whereas other GPI proteins such as Gas1p remain anchored in the plasma membrane, and cannot be

detected by antibodies on intact cells. Since the negative immunoselection enriched not only for *gpi* mutants but, a priori, also selected for mutants in signal transduction via the a-factor receptor, transcription, translation, translocation, secretion, and cell-wall integration of  $\alpha$ -agglutinin, it was necessary to perform additional screens. By eliminating mutants unable to induce and secrete acid phosphatase at 37°C, mutants deficient in transcription, translation, translocation, and secretion are removed, and theoretically one is left with mutants deficient in GPI anchoring, a-factor receptor signaling, and cell-wall integration. Among those, our screen detected *gpi* mutants at a frequency of 1:70, and we have not tested whether the other mutants really are deficient in signaling or cell-wall integration. It is somewhat difficult to estimate the efficiency of our selection procedure. When screening for colonies that fail to incorporate [ $2\text{-}^3\text{H}$ ]Ins into proteins, Leidich et al. (33) isolated five mutants from 25,000 colonies tested, but it remains uncertain if any of our mutants would have scored in their screen, since none of our mutants seems to be deficient in [ $2\text{-}^3\text{H}$ ]Ins incorporation into proteins to the same extent as the mutants isolated by Leidich et al. (33). On the other hand, it is quite conceivable that our screening procedure selected for leaky mutants, since a mutant that rapidly dies at 37°C would probably not have scored as a secretor in the 2-d plate assay for secretion of acid phosphatase. Yet, we believe that GPI anchoring is affected in all of our mutants, since all but the *gpi9* mutant accumulate a well-defined GPI lipid intermediate, suggesting that one specific step of the pathway has become rate limiting in each mutant. Such defects are compatible with the fact that all of the mutants are recessive, and that among the mutants that have a growth defect at 37°C, the *ts* growth phenotype cosegregates with the accumulation of an abnormal lipid. By testing a large panel of known mutants, we also could exclude that the accumulation of specific GPI lipids occurred as an indirect consequence of some other perturbation of the secretory pathway; neither secretion mutants (*sec1* to *sec23*) (16, 52, 61) nor ER or Golgi-retention mutants (*erd1*, *pnr1*, *anp1*, *van1*) (6, 22, 66), nor mutants in *N*-glycosylation (*alg2*, *alg3*) (27), nor mutants in *N*- or *O*-glycan elongation (*mnn1*, *mnn2*, *mnn3*, *mnn5*, *mnn9*, *mnt1*) (1, 23), nor in protein synthesis (*prt1-1*) accumulated any abnormal [ $2\text{-}^3\text{H}$ ]Ins-labeled lipids (Sipos, G., and M. Benghezal, unpublished data). In fact, the only glycosylation mutants that accumulate abnormal GPI intermediates are some mutants with defects in mannose metabolism (*sec53*, *sec59*, *dpm1*, *pni40*) and some of our mutants which accumulate GlcN-acylPI. It is noteworthy that none of the 800 isolates tested exhibited any defect in the biosynthesis of PI, inositolphosphoceramides, mannosylinositolphosphoceramide, or inositolphosphomannosylinositolphosphoceramide (61, 71), a finding that argues that our selection procedure enriched for GPI mutants, or else selected against defects in the biosynthesis of these phosphoinositides.

What are the steps which are blocked in the various mutants? Several mutants (*gpi4-gpi6*, *gpi9*) accumulate a GlcN-acylinositol-lipid intermediate, and therefore might have a defect in the  $\alpha$ 1,4-mannosyltransferase adding the first mannose of the GPI core structure or in the generation of dolicholphosphomannose required for this trans-

ferase (42, 55). Although both elongation of *N*-glycans and GPI biosynthesis are compromised in some of these mutants, the defect may well concern biosynthesis and transport of dolicholphosphomannose, rather than biosynthesis of GDP-mannose, since dolicholphosphomannose seems to be required for both of these processes. In fact, *dpm1*, a ts mutant in dolicholphosphomannose-synthase, has been shown to make (55) and to secrete (Benghezal, M., unpublished results) a severely underglycosylated invertase. This suggests that Och1p (50, 51) or subsequent elongating Golgi mannosyltransferases depend on the presence of some dolicholphosphomannose-derived core mannoses. Hence, the presence of some invertase with slightly or drastically increased mobility in *gpi4*, *gpi6*, and *gpi9* at 37°C (Fig. 11) might be indicative of some difficulty in making dolicholphosphomannose. Isolate 839 and *gpi7* might be deficient in the  $\alpha$ 1,2-mannosyltransferase adding the third mannose and the phosphoethanolamine group of the GPI core structure, respectively. 839 and *gpi7* might be in genes homologous to the mammalian PIG-B and PIG-F (29, 31). *Gpi8* might be deficient in the hypothetical transamidase that attaches CP's to GPI proteins or in a flippase that transfers CP's from the cytosolic to the luminal side of the ER (76, 77). At the moment, direct assays to measure these activities in microsomes have yet to be developed. Recently, *gaa1*, a ts mutant with a phenotype similar to *gpi8*, has been described (20). We find that *GPI8* and *GAA1* are different genes since *gpi8* and *gaa1* mutants are complementing each other and are synthetic lethal when combined in a single haploid strain. Also, transfection of the *GAA1* gene on a multicopy vector does not cure the defects of *gpi8* cells (not shown). Although not all mutants are ts for growth, cloning of the various *gpi* genes should be aided by the fact that all mutants are defective for growth on CFW at 37°C.

Some mutants accumulate severely reduced amounts of [2-<sup>3</sup>H]Ins-labeled proteins, but don't accumulate large amounts of abnormal lipids, whereas others accumulate large amounts of abnormal lipids, but are less severely affected in the incorporation of [2-<sup>3</sup>H]Ins into proteins. Mutants of both types can even be found among the various alleles of a single complementation class, *gpi7*. To explain this paradox, several possibilities might be considered: (a) It should be kept in mind that our pulse-labeling protocol does not label proteins and lipids to equilibrium, and that the observed accumulation of abnormal lipids is the result of both, the rate of biosynthesis as well as the rate of breakdown. Also, the rate of lipid accumulation of abnormal lipids may have been underestimated in mutants in which the decay of the mutated enzyme activity at 37°C is slow. (b) The same caveats make it impossible to take the reduction in [2-<sup>3</sup>H]Ins labeling of proteins as a measure of the reduction of GPI protein biosynthesis. In fact, the half-life of label on many GPI proteins is short (9), possibly because the phosphoinositide is lost from proteins getting incorporated into the cell wall (35), or because the lipid moiety of some GPI proteins are cleaved off at the plasma membrane and the proteins are lost into the culture medium (49). Thus, even in the presence of a significant reduction of incorporation of [2-<sup>3</sup>H]Ins into proteins, the amount of labeled protein accumulated during the 1.5-h labeling period may appear normal, if the mutation results

in a slowing down of vesicular traffic, and consequently the pool size of labeled proteins in the secretory pathway is increased. (c) Recent data from *Leishmania major* suggests that GPI's might be synthesized on both sides of the ER membrane by two separate biosynthetic pathways (44). If this were the case in yeast, it also might explain why some of our mutants accumulate high amounts of GPI lipids but are still able to add GPI anchors to proteins. (d) Mutations that increase the Michaelis constant ( $K_m$ ) of a given biosynthetic enzyme for its lipid substrate but don't alter its maximum velocity are expected to cause a relatively major increase of an abnormal GPI intermediate but to have less influence on the rate of GPI anchor biosynthesis. (e) GPI's might be synthesized by a multienzyme complex forming an assembly line in which substrates are transferred from one enzyme to the next without having to diffuse freely in the membrane. In this case, a mutation that renders a given enzyme of the assembly line rate limiting might lead to a significant reduction of GPI biosynthesis without causing the accumulation of an abnormal lipid, as long as the GPI intermediates proximal to the blocked enzyme cannot leave the assembly line. On the other hand, mutations affecting the interaction of enzymes of the multienzyme complex might disrupt the complex and lead to the accumulation of abnormal lipids that now have to reach the following enzyme by diffusion. (f) Mutations might also render enzymes insensitive to some negative feedback control, which might coordinate the amount of GPI biosynthesis with the rate of GPI protein synthesis.

The increased sensitivity of *gpi* mutants to CFW and hygromycin B suggests that GPI-anchored proteins are required for the integrity of the yeast cell wall. The requirement of GPI anchoring for cell-wall integrity has also been documented recently by the group of Frans Klis. Deletion of Gas1p results in CFW hypersensitivity (63) and *cwh6*, a CFW-hypersensitive mutant, has a defect in the yeast homologue of PIG-A, a gene required for the first step of GPI biosynthesis, namely the addition of GlcNAc onto PI (78).

It appears that our mutant isolation screen is by no means exhaustive, since many mutants were only obtained once. In addition, several intermediates of the GPI pathway such as GlcNAc-PI, GlcN-PI, and GlcN-acylinositol-P-lipid would not have been detected since they are expected to comigrate on TLC with PI and inositolphosphoceramides. We currently use slight modifications of this screening protocol in trying to obtain additional *gpi* mutants.

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