Saccharomyces cerevisiae GPI10, the functional homologue of human PIG-B, is required for glycosylphosphatidylinositol-anchor synthesis

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An increasing number of plasma membrane proteins have been shown to be attached to the membrane via a glycosylphosphatidylinositol (GPI) moiety. All eukaryotes share a highly conserved GPI-core structure EthN-P-Man₆GlcN-PI, where EthN is ethanolamine. We have identified a protein encoded by the yeast open reading frame YGL142C that shares 33% identity with the human Pig-B protein. Deletion of this essential gene leads to a block in GPI anchor biosynthesis. We therefore named the gene GPI10. Gpi10p and Pig-B are functional homologues and the lethal deletion of GPI10 can be rescued by expression of the PIG-B cDNA. As found for PIG-B mutant cells, gpi10 deletant cells cannot attach the third mannose in an α₁,2 linkage to the GPI core-structure intermediate. Overexpression of GPI10 gives partial resistance to the GPI-synthesis inhibitor YW3548, suggesting that this gene product may affect the target of the inhibitor.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) anchoring represents a mechanism for attachment of proteins to membranes found in all eukaryotic cells [1,2]. All eukaryotes share the common GPI core structure Eth₁₆Man₁₂,6Man₁₄,1,6-Myo-inositol-P-lipid, where Eth₁₆ is ethanolamine. Modifications of this core structure can occur on different mannose residues and depends on the species as well as on its developmental stage. The lipid moiety of the GPI anchor is also subject to modifications, such as variations of the fatty acids, ceramide remodelling or the attachment of alkyl chains [1].

The complete GPI precursor, whose structure has been solved for some species, is assumed to be assembled by sequential addition of the sugar components and ethanolamine to phosphatidylinositol [3]. The complete precursor is transferred en bloc to the anchor-attachment site of the protein (ω-site) with concomitant release of a C-terminal peptide [4]. Anchor attachment is thought to occur as a transamidation reaction [5,6]. A protein to be GPI-anchored carries two signal sequences: a cleavable N-terminal signal sequence that causes the protein to be translocated into the lumen of the endoplasmic reticulum (ER), and a C-terminal signal sequence that directs attachment of the GPI anchor. The consensus sequence for GPI-anchor addition consists of an amino acid with a small side-chain at the ω-site, two amino acids with small side-chains in positions ω + 1 and ω + 2, and a short hydrophilic spacer region followed by a hydrophobic domain of about 15–20 amino acids [7].

The identification of at least eight complementation classes of mutants from murine lymphoma and human K562 cells, lacking the expression of GPI-anchored proteins at the cell surface, led to the characterization of genes involved in mammalian anchor biosynthesis [3]. Three complementation classes, A, C and H, are blocked in the first step of precursor synthesis, which is the addition of GlcNac to PI [8]. Class J mutants and class L mutants are defective in deacetylating GlcNac-PI to give GlcN-PI [9–11]. Class E mutants are defective in dolichyl (Dol)-P-Man synthase, thereby affecting GPI mannosylation and N-linked glycosylation [12]. Class B mutant cells are blocked in the addition of the third mannose to the intermediate structure [13], the class F mutation affects the transfer of the terminal ethanolamine to the third mannose [12], and class K mutants accumulate the complete precursor without transferring it to the protein [10]. Whereas in mammalian cells, several cDNAs that encode components of the GPI synthesis machinery (PIG-A, PIG-B, PIG-L, PIG-F, PIG-C and PIG-B) have been cloned [11,13–17], only genes involved in the first step of anchor synthesis (PIG1, PIG2, PIG3, SPT13) and in anchor attachment (GAA1, GPI8) have been identified from yeast [18–21]. Yeast mutants that affect various steps of GPI-anchor synthesis have also been described [22].

The core structure of the GPI anchor contains three mannose residues which are all assumed to be transferred from Dol-P-Man as donor [23]. The mannose residues were found to be attached in different linkages, suggesting that at least three gene products are involved in GPI mannosylation. The Pig-B protein is required for the transfer of the third mannose in an α₁,2 linkage to the core structure, but to date, no enzymic activity of the Pig-B protein has been shown [13]. A yeast mutant, blocking a similar step as the Pig-B mutant, has been isolated [22]. Addition of the third mannose can also be blocked using a species-specific inhibitor, YW3548 [24]. YW3548 blocks GPI-anchor synthesis in mammalian cells and in yeast, but not in protozoa.

In this study, we report the identification of a yeast gene, GPI10, which appears to be the functional homologue of the human PIG-B gene. In order to study the function of this essential gene, we placed GPI10 under the control of the GAL1/10 promoter. When GPI10 expression was turned off, we found that mannosyl was no longer incorporated into proteins and that the maturation of Gas1p, a major GPI-anchored protein from yeast, was strongly reduced. Under these conditions, we observed the accumulation of the GPI intermediate Man₆GlcN-(acyl)PI, and of a novel yeast GPI-derived structure that was sensitive to treatment with HF and to Jack bean mannosidase.

Abbreviations used: ER, endoplasmic reticulum; EthN, ethanolamine; GPI, glycosylphosphatidylinositol; GU, glucose unit.

1 To whom correspondence should be addressed.
most likely Man-(EthN-P)Man-GlcN-(acetyl)PI. The appearance of this novel lipid was blocked by addition of YWW3548, a GPI-synthesis inhibitor. Overexpression of GPI10 rendered wild-type cells partially resistant to YWW3548.

MATERIALS AND METHODS

Strains and growth conditions

Cells were grown to saturation in either YPG [3 % (v/v) glycerol, 2 % (w/v) peptone, 1 % (w/v) yeast extract, 40 mg/l each of adenine, uracil and tryptophan] or in SGYE [3 % (v/v) glycerol, 0.67 % (w/v) yeast nitrogen base, 0.2 % (w/v) yeast extract and the required nutrients] and used to inoculate glucose-0.67 % acetate, 2 % Bacto-agar, and the required nutrients to complement auxotrophs.

The full-length sequence of GPI10 was replaced by the KanMX module using a PCR-based strategy [25]. A knockout cassette was constructed using a short flanking homology strategy and the oligomers: (upstream) 5′ TCTCTCACAGTATGGTCGTCT- GATTATTATATTACTACTGCCGAACGTACGCTGC- GTAAGTCTGTATATTACGATAGGGTCT- TCTCTCAAG 3′ and (downstream) 5′ ATCGATGAATT- CAGCGTGACC 3′ and (downstream) 5′ ATCGATGAATT- CAGCGTGACC 3′ and (downstream) 5′ ATCGATGAATT- CAGCGTGACC 3′ and (downstream) 5′ ATCGATGAATT-

Plasmids

pGal-GPI10 was constructed by PCR amplification of the full-length GPI10 coding sequence from genomic DNA, with the primers (upstream) 5′ GATAGTCTAGAATTTGTAACCAA 3′ and (downstream) 5′ GTAAACTGATAGTGTAATTACGATAGGGTCT- TCTCTCAAG 3′ and (downstream) 5′ ATCGATGAATT-

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<td>Eurofan</td>
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<td>Cen.PK2</td>
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[H]myo-Inositol labelling of proteins

Wild-type cells or gpi10A::KanMX cells carrying pGal-GPI10 were grown in SGYE to saturation and used to inoculate SDYE (5 % glucose) in which they were grown for about 16 h. Cells (5 x 10⁹) were washed twice in SD-inositol medium (5 % glucose) [26], resuspended in 500 µl of SD without inositol (5 % glucose) and depleted of inositol for 10 min before the addition of 15 µCi of [H]myo-inositol (Du Pont de Nemours, Bad Homburg, Germany). Cells were labelled for 30 min. A total protein extract was prepared by lysing the cells in TEPI [100 mM Tris/HCl, pH 7.5/10 mM EDTA/protease inhibitors (1 µg/ml peptatin, 1 µg/ml leupeptin, 1 µg/ml antipain)] by vortexing for 4 x 1 min with glass beads. Total protein was precipitated with trichloroacetic acid and the precipitates were washed with acetone and resuspended in Laemmli protein sample buffer. The proteins were separated by SDS/PAGE (10 % acrylamide) and, after incubation of the gel in 1 M sodium salicylate, the radioactivity was detected by fluorography.

Pulse–chase analysis of Gas1p and CPY

Pulse-chase labelling and analysis of immunoprecipitates was done as described previously [26]. Briefly, wild-type cells and gpi10A::KanMX cells carrying pGal-GPI10 were grown to saturation in SGYE and shifted to SDYE (5 % glucose) for 16 h. Cells were harvested and washed with SD* [5 % (w/v) glucose, 0.67 % (w/v) yeast nitrogen base and the required nutrients]. Cells (2.5 x 10⁶ per time point) were resuspended in 1 ml of SD*, preincubated for 10 min, labelled with TRANS²⁵S-label (Dupont de Nemours) and chased for the indicated times with 0.003 % (w/v) methionine/0.003 % (w/v) cysteine/3 mM (NH₄)₂SO₄. Aliquots were taken and the chase was stopped by adding NaN₃ and NaF to a final concentration of 10 mM. Cells were lysed by vortexing for 4 x 1 min with glass beads in TEPI. The lysates were boiled in the presence of 1 % SDS for 5 min and centrifuged for 15 min in an Eppendorf centrifuge. The supernatant was added to 5 ml of TNET (100 mM Tris/HCl, pH 8/100 mM NaCl/5 mM EDTA/1 % Triton X-100) and the extracts were incubated with polyclonal antisera against Gas1p or CPY and...
The resulting fragment was desalted and purified on a G-15 with Jack bean mannosidase (Sigma) for 40 h with 2 mM EDTA to convert the non-hydrophilic fragment into hydrophilic fragments or core glycans by desalting by phase partitioning between n-butanol and 0.1 mM CHCl₃/HCl, pH 7.5, and analysed by TLC using Bio-Gel P4 or high-pH anion-exchange TLC plate, respectively, as described previously [28,29].

**Glycolipid labelling in vivo**

[¹⁴C]Mannose labelling experiments in vivo using RH3998 and RH3999 were performed as described [27]. In brief, pmi40 or pm140 gpi10Δ::KanMX cells carrying the plasmid pGal-GPI10 were grown for 16 h in SDCU medium [5% (w/v) glucose/1% (w/v) peptone/0.67% (w/v) yeast nitrogen base/0.1% (w/v) mannose, supplemented with 40 mg/l uracil]. Cells (3 × 10⁸) were resuspended in SPCU medium [0.1% (w/v) glucose, 2% (w/v) pyruvate, 0.67% (w/v) yeast nitrogen base and the required nutrients], preincubated at a non-permissive temperature for pmi40 for 20 min and labelled with 25 μCi of [¹⁴C]mannose for 45 min. In some experiments, cells were incubated with 5 μg/ml YW3548 (Novartis AG, Basel, Switzerland) for 10 min before addition of the radioactive reaction. The reaction was stopped by the addition of 10 mM NaF/10 mM NaN₃, and lipids were extracted with CHCl₃/CH₃OH/H₂O (10:10:3, by vol.). Lipids were desalted by phase partitioning between n-butanol and 0.1 mM EDTA/5 mM Tris/HCl, pH 7.5, and analysed by TLC using CHCl₃/CH₃OH/H₂O (10:10:3, by vol.) as solvent. For structural analysis, the accumulated lipids were extracted from the TLC plate, converted into hydrophilic fragments or core glycans by Bio-Gel P4 or high-pH anion-exchange chromatography respectively, as described previously [28,29]. The hydrophilic fragment generated using HNO₃ digestion with Jack bean mannosidase (Sigma) for 40 h with 2 × 1.5 units. The resulting fragment was desalted and purified on a G-15 column, then analysed on Dionex as above.

**Halo assay**

RH1657 cells expressing either pGal or pGal-GPI10 were grown to saturation in YGal medium. Cells (5 × 10⁸) were included in 10 ml agar plates (0.8% agar) of the same media and 4 μl of YW3548 at different concentrations was spotted on the plate.

**RESULTS**

**Identification of GPI10**

A database search using the National Center of Biotechnology Information BLAST server indicated a large extent of homology between the protein encoded by the yeast open reading frame YL142C and the human Pig-B protein. Owing to the results presented below, we named this gene GPI10. The GPI10 gene encodes a protein of 616 amino acids with a predicted size of about 72 kDa. It shows 33% identity with and 58% similarity to the human PIG-B gene (Figure 1), the potential third mannosyltransferase producing the GPI anchor. In addition, the hydrophobicity profiles of the two proteins are very similar (results not shown).

**GPI10 is essential and its deletion can be rescued by expression of the human PIG-B cDNA**

To test whether GPI10 is an essential gene, we constructed a KanMX knockout cassette and transformed it into the diploid wild-type strain Cen.PK2 to disrupt the GPI10 gene [25]. Upon tetrad dissection, we found that only two spores from each tetrad could grow (Figure 2A). The growing spores were sensitive to geneticin, showing that spores that were deleted for GPI10 were inviable. GPI10 was also found to be essential in other strain backgrounds (results not shown).

To study the function of the GPI10 gene product, we constructed a conditional mutant of GPI10 by cloning the gene under control of the GAL1/10 promoter on a centromeric plasmid. In this way, the expression of GPI10 can be turned off simply by shifting the cells from glycerol-containing medium (SGYE or YPG), in which the promoter is not repressed, to glucose-containing medium (SDYE or YPD, both containing 5% glucose) where it is. The Gal construct of GPI10 (pGal-GPI10) was functional and could restore growth of gpi10Δ::KanMX spores (Figure 2B). This construct was used to perform Gpi10p depletion studies.

GPI10 had been identified based on its high degree of homology to the human PIG-B gene. We therefore tested whether GPI10 could be functionally replaced by PIG-B. PIG-B was cloned into a yeast multicopy vector (p425) behind the MET promoter, which is activated in the absence of methionine. p425-PIG-B was transformed into the Cen.PK2 diploid strain which is heterozygous for gpi10Δ::KanMX. The diploid was sporulated on minimal sporulation plates and tetrads were dissected onto minimal medium plates without methionine. Despite low overall spore viability, we obtained spores that carried the plasmid and were grown (Figure 2A). The growing spores were sensitive to geneticin, showing that spores that were deleted for GPI10 were inviable (Figure 2B). This construct was used to perform Gpi10p depletion studies.

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The sequence alignment was done using the BESTFIT program of the Wisconsin Package Version 9.0, Genetics Computer Group (GCC), Madison, WI, U.S.A. Identical residues are shown in boxes.

**Figure 1** Sequence alignment of Gpi10p and the Pig-B protein

The sequence alignment was done using the BESTFIT program of the Wisconsin Package Version 9.0, Genetics Computer Group (GCC), Madison, WI, U.S.A. Identical residues are shown in boxes.
which were resistant to geneticin (Figure 2C), suggesting that they were deleted for GPI10. PIG-B expression did not completely restore wild-type growth levels in gpi10Δ::KanMX strains [compare spore A (gpi10Δ::KanMX) and spore B (wild type) in Figure 2C], but partial restoration of phenotypes is commonly seen for functional complementation over such a large evolutionary distance. Expression of PIG-B from a centromeric vector was not sufficient to restore growth (results not shown). We conclude that expression of the human PIG-B cDNA can complement the lethal phenotype of gpi10Δ::KanMX cells, suggesting that the two proteins are functional homologues.

Gpi10p depletion results in a GPI-synthesis defect
The GAL1/10-controlled GPI10 vector allowed us to regulate the expression of GPI10. To find conditions where Gpi10p was functionally depleted, we followed the growth of the spores of a tetrad (Figure 2B) upon shift from glycerol- to glucose-containing medium. As shown in Figure 3(A), the growth of gpi10Δ::KanMX cells slowed down significantly after about 8 h incubation in glucose-containing medium, suggesting that Gpi10p was strongly depleted. For the following experiments, we used conditions where GPI10 expression was turned off for 8–16 h. We had also tried to shift cells from galactose- to glucose-containing medium, but observed no change in growth rate over a period of 24 h (results not shown). This could be due to a very high expression of Gpi1p on galactose medium combined with a long half-life of the protein.

Since GPI10 appears to be the functional homologue of PIG-B, we tested whether GPI10 is indeed involved in GPI-anchor synthesis. GPI-anchored proteins are the only proteins that incorporate [3H]myo-inositol due to the inositol residue in the GPI anchor. Cells were grown in SGEY to saturation and used to inoculate SDYE (5% glucose). After 16 h growth in SDYE (5% glucose) the cells were labelled for 30 min with [3H]myo-inositol, total protein was extracted and analysed by SDS/PAGE, followed by fluorography. We observed that in gpi10Δ::KanMX spores (B and D), the incorporation of radiolabelled inositol was greatly reduced when compared with the wild-type spores (A and C), demonstrating a defect in GPI-anchoring (Figure 3B). Furthermore, pulse-chase analysis of the GPI-anchored protein Gas1p was performed. Gas1p occurs as a 105 kDa form in the ER. Upon arrival at the Golgi, its core glycans are elongated, causing a shift in apparent molecular mass from 105 to 125 kDa. Transport of Gas1p from the ER to the Golgi depends on addition of the GPI anchor [30,31]. After 16 h of shift from SGEY medium to SDYE medium (5% glucose), cells were labelled for 5 min and chased for various amounts of time. Cells were lysed and either Gas1p or the non-GPI-anchored vacuolar hydrolase carboxypeptidase Y was precipitated from total lysates using polyclonal antisera. Immune complexes were separated by SDS/PAGE and analysed on a PhosphorImager. As shown in...
and of a novel lipid GlcN-PI \[32\]. To test if mannose to the intermediate structure Man-(EthN-P)Man-GlcN-(acyl)PI in GPI-anchor synthesis.

transport defect. These data are consistent with a role for Gpi10p

Therefore, the defect in maturation does not represent a general

labelling experiments

in vivo. Wild-type yeast cells incorporate little exogenously added mannose. We therefore used cells carrying the pmi40 mutation, which is a temperature-sensitive

allele of phosphomannose isomerase. Upon shift to non-permissive temperature, these cells incorporate exogenously added mannose with high efficiency. The pmi40 mutation was introduced into a gpi10A::KanMX strain containing pGal-GPI10 (RH3999). A pmi40 GPI10 strain derived from the same cross was used as control (RH3998). Cells were grown to saturation in YPG and shifted to SDCU medium (5% glucose) for 16 h. Cells were preincubated for 10 min in the presence or absence of the GPI-synthesis inhibitor, YW3548, which causes the accumulation of Man$_3$-GlcN-(acyl)PI \[24\]. After labelling for 45 min with [14C]mannose, total lipids were extracted and analysed by TLC (Figure 4). In wild-type cells, one can see some labelling of the complete GPI precursor and mannosylated sphingolipids. When the inhibitor YW3548 was added, a strong accumulation of a band comigrating with Man$_3$-GlcN-(acyl)PI occurred \[24\]. In Gpi10p-depleted cells, Man$_3$-GlcN-(acyl)PI accumulated even in the absence of YW3548. In addition, a more polar lipid (indicated with an arrow in Figure 4) was strongly labelled, which was sensitive to treatment with GPI phospholipase D, but resistant to digestion with PI phospholipase C, suggesting that the lipid had an acylated inositol ring (results not shown).

The appearance of this lipid is strongly diminished by YW3548. From the novel lipid and from the Man$_3$-GlcN-(acyl)PI, hydrophilic fragments were prepared by nitrous acid deamination and borohydride reduction and were analysed by Bio-Gel P4 chromatography. Furthermore, neutral glycans were prepared by treatment with HF, followed by nitrous acid deamination and borohydride reduction, and were analysed by Dionex chromatography (Table 2). In addition, the deaminated, reduced fragment derived from the novel lipid was subjected to treatment with Jack bean mannosidase followed by analysis by Dionex chromatography (Table 2). The novel lipid is sensitive to treatment with HF, contains the core glycan Man$_2$-anhydromannitol and elutes from a Bio-Gel P4 sizing column at 7.3 glucose units (GU). On the same column, a deaminated, reduced fragment derived from T. brucei lipid C (EthN-P-Man$_2$-GlcN-PI) eluted at 7.8 GU. Jack bean mannosidase treatment resulted in a shift in the elution profile from 7.3 to 7.0 GU and the release of a fragment eluting at 0.9 GU (mannose). These findings are consistent with a structure Man-(EthN-P)Man-GlcN-(acyl)-PI. One additional ethanolamine phosphate leads to an apparent size increase of about 4 GU as previously demonstrated by comparing the elution position of EthN-P-Man$_2$-GlcN-PI and Man$_3$-GlcN-PI \[28\].

**Table 2** Analysis of the lipid intermediates accumulated upon depletion of Gpi10p

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<th>Lipid</th>
<th>Bio-Gel P4 Gel filtration analysis of the hydrophilic fragments (GU)</th>
<th>Dionex HPAEC analysis of the neutral core glycans (DU)</th>
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<td>Man$_3$-GlcN-(acyl)PI</td>
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<tr>
<td>Novel lipid</td>
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<tr>
<td>Novel lipid (JBAM)</td>
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Figure 3(C), Gas1p maturation was severely delayed in gpi10A::KanMX spores (B and D) when compared with wild-type spores (A and C). Under these conditions, maturation of carboxypeptidase Y was normal in all four spores, showing that its transport to the vacuole was unaffected (results not shown). Therefore, the defect in maturation does not represent a general transport defect. These data are consistent with a role for Gpi10p in GPI-anchor synthesis.

Pig-B mutant cells are defective for the addition of the third mannose to the intermediate structure Man-(EthN-P)Man$_3$-GlcN-PI \[32\]. To test if GPI10 also mediates the addition of the third mannose to the GPI intermediate, we performed lipid labelling experiments in vivo. Wild-type yeast cells incorporate little exogenously added mannose. We therefore used cells carrying the pmi40 mutation, which is a temperature-sensitive

allele of phosphomannose isomerase. Upon shift to non-permissive temperature, these cells incorporate exogenously added mannose with high efficiency. The pmi40 mutation was introduced into a gpi10A::KanMX strain containing pGal-GPI10 (RH3999). A pmi40 GPI10 strain derived from the same cross was used as control (RH3998). Cells were grown to saturation in YPG and shifted to SDCU medium (5% glucose) for 16 h. Cells were preincubated for 10 min in the presence or absence of the GPI-synthesis inhibitor, YW3548, which causes the accumulation of Man$_3$-GlcN-(acyl)PI \[24\]. After labelling for 45 min with [14C]mannose, total lipids were extracted and analysed by TLC (Figure 4). In wild-type cells, one can see some labelling of the complete GPI precursor and mannosylated sphingolipids. When the inhibitor YW3548 was added, a strong accumulation of a band comigrating with Man$_3$-GlcN-(acyl)PI occurred \[24\]. In Gpi10p-depleted cells, Man$_3$-GlcN-(acyl)PI accumulated even in the absence of YW3548. In addition, a more polar lipid (indicated with an arrow in Figure 4) was strongly labelled, which was sensitive to treatment with GPI phospholipase D, but resistant to digestion with PI phospholipase C, suggesting that the lipid had an acylated inositol ring (results not shown). The appearance of this lipid is strongly diminished by YW3548. From the novel lipid and from the Man$_3$-GlcN-(acyl)PI, hydrophilic fragments were prepared by nitrous acid deamination and borohydride reduction and were analysed by Bio-Gel P4 chromatography. Furthermore, neutral glycans were prepared by treatment with HF, followed by nitrous acid deamination and borohydride reduction, and were analysed by Dionex chromatography (Table 2). In addition, the deaminated, reduced fragment derived from the novel lipid was subjected to treatment with Jack bean mannosidase followed by analysis by Dionex chromatography (Table 2). The novel lipid is sensitive to treatment with HF, contains the core glycan Man$_2$-anhydromannitol and elutes from a Bio-Gel P4 sizing column at 7.3 glucose units (GU). On the same column, a deaminated, reduced fragment derived from T. brucei lipid C (EthN-P-Man$_2$-GlcN-PI) eluted at 7.8 GU. Jack bean mannosidase treatment resulted in a shift in the elution profile from 7.3 to 7.0 GU and the release of a fragment eluting at 0.9 GU (mannose). These findings are consistent with a structure Man-(EthN-P)Man-GlcN-(acyl)-PI. One additional ethanolamine phosphate leads to an apparent size increase of about 4 GU as previously demonstrated by comparing the elution position of EthN-P-Man$_2$-GlcN-PI and Man$_3$-GlcN-PI \[28\].

**GPI10 overexpression results in partial resistance to YW3548**

As YW3548 prevents the addition of the third mannose to the GPI intermediate Man$_3$-GlcN-(acyl)PI and our data suggest that

Figure 4 Gpi10p depletion causes the accumulation of Man$_3$-GlcN-(acyl)PI and of a novel lipid

pmi40 (RH3998) or pmi40 gpi10A::KanMX (RH3999), both carrying pGal-GPI10, were shifted to glucose-containing medium for 16 h. Cells were labelled for 45 min with [14C]mannose, followed by extraction of total lipids and analysis by TLC. Man$_2$, Man$_3$-GlcN-(acyl)PI; MIPC, mannosylinositolphosphorylceramide; M(IP)$_2$C, mannosyl-diinositolphosphorylceramide; arrow, novel lipid; CP, complete precursor; O, origin.
Both genes appear to mediate the same step in GPI-anchor synthesis. Previous studies have indicated a high degree of homology between the mammalian and the yeast GPI-anchor synthesis machinery. The gpi8 ts mutant was shown to be complemented by its human homologue [21]. Also, mammalian class E mutants that are defective in Dol-P-Man synthase can be complemented by the homologous yeast gene [33]. In this study we show that a complete deletion of GPI10 can be complemented by its human homologue, the PIG-B gene.

As expected from the sequence homology and the functional complementation test, GPI10 is involved in GPI-anchor synthesis. When GPI10 expression was repressed, radiolabelled inositol incorporation into proteins was greatly reduced and Gas1p maturation was strongly delayed, which is consistent with a GPI-synthesis defect. All other genes that have been implicated in yeast GPI-anchor synthesis have also been found to be essential [18–22,34].

When GPI10 expression was turned off, Man$_\alpha$GlcN-(acyl)PI as well as a novel lipid were found to accumulate. This lipid contained a Man$_\alpha$-anhidromannitol core glycan after dephosphorylation, nitrous acid treatment and reduction. Its hydrophilic fragment, however, eluted from a Bio-Gel P4 column at a much later position, which could be due to an ethanolamine phosphate attached to Man$_\alpha$-anhidromannitol. Furthermore, it is sensitive to treatment with Jack bean mannosidase. These findings suggest that the novel lipid may represent a GPI intermediate, most likely Man-(EthN-P)Man-GlcN-(acyl)PI. For the moment there is no formal proof that the aqueous HF-sensitive group on the mannose is ethanolamine phosphate. At this point different possibilities exist: it could represent an aberrant structure, which is only synthesized due to extensive pressure on the GPI-synthesis machinery imposed by the depletion of Gpi10p, or it could represent a natural intermediate. Most GPI anchors isolated from mature yeast proteins do not seem to carry an ethanolamine phosphate modification on the first mannose [35]. However, in animal cells, GPI molecules have been identified with this modification [1], and the mammalian PIG-B enzyme most likely acts on a GPI precursor with the same carbohydrate structure that accumulated in this study. Previous studies, which characterized the complete, radiolabelled GPI precursor in yeast before it was added to the protein, would not necessarily have detected this modification with the techniques used [27].

Previously, we suggested that YW3548 blocked the addition of the third mannose to the GPI core because when cells or membranes were treated with the compound, a Man$_\alpha$-GPI intermediate with two mannoses and no ethanolamine accumulated. Here we have observed that the addition of a putative ethanolamine phosphate to the Man$_\alpha$-GPI intermediate was prevented by incubation of cells with YW3548. This raises the possibility that the inhibition of the addition of the third mannose to the GPI core is indirect and is due to the inhibition of ethanolamine phosphate addition to the first mannose by YW3548. Man-(EthN-P)Man-Glc-(acyl)PI could as well be a novel lipid to accumulate. This lipid contained a Man$_\alpha$-anhidromannitol core glycan after dephosphorylation, nitrous acid treatment and reduction. Its hydrophilic fragment, however, eluted from a Bio-Gel P4 column at a much later position, which could be due to an ethanolamine phosphate attached to Man$_\alpha$-anhidromannitol. Furthermore, it is sensitive to treatment with Jack bean mannosidase. These findings suggest that the novel lipid may represent a GPI intermediate, most likely Man-(EthN-P)Man-GlcN-(acyl)PI. For the moment there is no formal proof that the aqueous HF-sensitive group on the mannose is ethanolamine phosphate. At this point different possibilities exist: it could represent an aberrant structure, which is only synthesized due to extensive pressure on the GPI-synthesis machinery imposed by the depletion of Gpi10p, or it could represent a natural intermediate. Most GPI anchors isolated from mature yeast proteins do not seem to carry an ethanolamine phosphate modification on the first mannose [35]. However, in animal cells, GPI molecules have been identified with this modification [1], and the mammalian PIG-B enzyme most likely acts on a GPI precursor with the same carbohydrate structure that accumulated in this study. Previous studies, which characterized the complete, radiolabelled GPI precursor in yeast before it was added to the protein, would not necessarily have detected this modification with the techniques used [27].

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also appears to have an EthN-P on the first mannos of the core GPI structure (C. Süßterlin, P. Gerold, R. Schwarz and H. Riezman, unpublished work). This newly proposed sequence of events in the yeast GPI synthesis pathway would also be consistent with the lack of activity of YW3548 on all protozoa tested [24], because these cells do not use this modification on the first mannos [1]. Previously, we suggested that YW3548 might mimic the Man$_2$GlCN-(acyl)PI substrate and compete for binding of this substrate to the α-1,2 mannosyltransferase [24]. This same argument would hold for the phosphoethanolamine transferase needed to generate this novel yeast lipid.

Overexpression of GPI10 can confer partial resistance to YW3548, as tested by growth in the presence of YW3548. The partial resistance could be explained if Gpi10p were one of the targets of YW3548 or if the real target is the phosphoethanolamine transferase. In the latter case, Gpi10p may utilize the unmodified Man$_2$GPI only poorly as a substrate. Although no mannosyltransferase activity of Gpi10p and the Pig-B protein have been shown, both proteins share significant homology with Alg9p (18% identity, 31% similarity). Alg9p is involved in the transfer of the seventh mannos in N-glycan precursor synthesis [36]. The mannos in that case is also donated by Dol-P-Man and is attached in an α-1,2 linkage. It is therefore likely that Pig-B, Gpi10p and Alg9p represent a new family of Dol-P-Man-requiring α-1,2 mannosyltransferases.

The identification of GPI10 as a gene involved in an intermediate step of GPI-anchor synthesis improves the characterization of the yeast GPI-synthesis machinery. Functional complementation could also allow the cloning of the protozoan enzymes which, at least in their native environments, have been shown not to be sensitive to YW3548 [24]. Expression of these genes in yeast may make it possible to screen for compounds that specifically interfere with the addition of the third mannose residue in fungi or protozoa.

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