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-Man₃-GlcN-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 α -1,2 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 EthN-P-6Man α -1,2Man α -1,6Man α 1,4-GlcN α -1,6-*myo*-inositol-P-lipid, where EthN 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 $\omega + 1$ and $\omega + 2$, 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-H*, *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 (*GPI1*, *GPI2*, *GPI3/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 α -1,2 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 inositol 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 Man₃-GlcN-(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.

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most likely Man-(EthN-P)Man-GlcN-(acyl)PI. The appearance of this novel lipid was blocked by addition of YW3548, a GPI-synthesis inhibitor. Overexpression of *GPI10* rendered wild-type cells partially resistant to YW3548.

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 the glucose-containing media SDYE [0.2 % (w/v) yeast extract, 0.67 % (w/v) yeast nitrogen base and 5 % (w/v) glucose] or YPUAD [1.0 % (w/v) yeast extract, 2 % (w/v) peptone, 5 % (w/v) glucose and 40 mg/l each of uracil and adenine]. For overexpression experiments, cells were grown in YGal medium [2 % (w/v) galactose, 1 % (w/v) yeast extract, 2 % (w/v) peptone, 40 mg/l of each of adenine, uracil and tryptophan]. Sporulation of yeast strains was on minimal sporulation medium (1 % potassium acetate, 2 % Bacto-agar, and the required nutrients to complement auxotrophies).

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' TCTCTCAACGATAGGGTCT-GATTTATTAATTTTACTACTGCCGAAACGTACGCTGC-AGGTCGACC 3' and (downstream) 5' ATCGATGAATTCGAGCTCGTTATGTAAGTTAATATCGCTATAAGGT-CGCCGTCTATAAATTTAAA 3'.

This cassette was transformed into the diploid strains FY1679 and CEN.PK2. Deletion of the gene was monitored by resistance to geneticin (Gibco-BRL) and resistant colonies were confirmed by PCR analysis. For overexpression studies from the *GAL1* promoter, RH1657 was used. For galactose-depletion studies, RH3699 was transformed with pGal-GPI10 and tetrads were dissected, generating spore A (RH3994), spore B (RH3995), spore C (RH3996) and spore D (RH3997). For *in vivo* mannose labelling experiments, RH3998 and RH3999 were used. All yeast strains used in this study are shown in Table 1.

Plasmids

pGal-GPI10 was constructed by PCR amplification of the full-length *GPI10* coding sequence from genomic DNA, with the primers (upstream) 5' GATAGTCTAGAATTGTGTAATC CAA 3' and (downstream) 5' GTAAACTGATAAGTGAAG-CATGCAC 3', using PFU Polymerase (Stratagene). The PCR

fragment containing sites for *XbaI* (upstream oligomer) and *SphI* (downstream oligomer) was subcloned into the *XbaI*-*SphI* site of the pSEYC68 vector containing the *GAL1/10* promoter cloned into the *EcoRI*-*BamHI* site (referred to as pGal).

p425-PIG-B was prepared by subcloning the full-length *PIG-B* cDNA [13] into the *EcoRV* site of pBluescriptII (Stratagene), and the fragment generated by cutting with *SpeI* and *SalI* was ligated into p425 digested with *SpeI* and *SalI*.

[³H]myo-Inositol labelling of proteins

Wild-type cells or *gpi10Δ::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 (5×10^7) were washed twice in SD-inositol medium (5 % glucose) [26], resuspended in 500 μ l of SD without inositol (5 % glucose) and depleted of inositol for 10 min before the addition of 15 μ Ci of [³H]myo-inositol (Dupont 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/proteinase inhibitors (1 μ g/ml pepstatin, 1 μ g/ml leupeptin, 1 μ g/ml antipain)] by vortexing for 4 $\times$ 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 *gpi10Δ::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 (2.5×10^7 per time point) were resuspended in 1 ml of SD*, preincubated for 10 min, labelled with TRANS ³⁵S-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 Na₂S₂O₃ and NaF to a final concentration of 10 mM. Cells were lysed by vortexing for 4 $\times$ 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

Table 1 Strains of *Saccharomyces cerevisiae* that were used for this study

Strain	Phenotype	Source
FY1679	<i>Mata</i> α <i>ura3-52/ura3-52 leu2Δ/ + trp1Δ63/ + his3Δ200/ +</i>	Eurofan
Cen.PK2	<i>Mata</i> α <i>ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289 his3Δ1/his3Δ1</i>	Eurofan
RH3698	FY1679, but <i>gpi10Δ::KanMX/GPI10</i>	This study
RH3699	Cen.PK2, but <i>gpi10Δ::KanMX/GPI10</i>	This study
RH3994	<i>Mata</i> <i>ura3-52 leu2-3,112 trp1-289 his3Δ</i> pGal-GPI10	This study
RH3995	<i>Mat</i> α <i>gpi10Δ::KanMX. ura3-52 leu2-3,112 trp1-289 his3Δ</i> pGal-GPI10	This study
RH3996	<i>Mata</i> <i>ura3-52 leu2-3,112 trp1-289 his3Δ</i> pGal-GPI10	This study
RH3997	<i>Mat</i> α <i>gpi10Δ::KanMX. ura3-52 leu2-3,112 trp1-289 his3Δ</i> pGal-GPI10	This study
RH3998	<i>Mat</i> α <i>pmi40 ura3 leu2 ade2 trp1 his4</i> pGal-GPI10	This study
RH3999	<i>Mat</i> α <i>pmi40 gpi10Δ::KanMX. ura3 leu2 ade2 trp1 his4</i> pGal-GPI10	This study
RH1657	<i>Mata</i> <i>his4 leu2 ura3 lys2 bar1</i>	Laboratory strain

Protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 3 h at room temperature. The immunoprecipitates were washed four times with TNET, once with 20 mM Tris, pH 7.4, resuspended in Laemmli sample buffer and analysed by SDS/PAGE, followed by exposure and quantification of the gel on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The percentage of mature GasIp was determined as the percentage of the total GasIp signal found in the 125 kDa form.

Glycolipid labelling *in vivo*

[¹⁴C]Mannose labelling experiments *in vivo* using RH3998 and RH3999 were performed as described [27]. In brief, *pmi40* or *pmi40 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 (3×10^7) 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 μ Ci of [¹⁴C]mannose for 45 min. In some experiments, cells were incubated with 5 μ g/ml YW3548 (Novartis AG, Basel, Switzerland) for 10 min before addition of the radioactivity. 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 and analysed by Bio-Gel P4 or high-pH anion-exchange chromatography respectively, as described previously [28,29]. The hydrophilic fragment generated using HNO₃ was digested 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 (5×10^6) were included in 10 ml agar plates (0.8 % agar) of the same media and 4 μ l 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 mannosyl-transferase 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

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 (GCG), Madison, WI, U.S.A. Identical residues are shown in boxes.

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

Figure 2 *GPI10* is essential for growth and its deletion can be complemented by pGal-GPI10 or by p425-PIG-B

(A) The *GPI10* open reading frame was replaced by KanMX in the diploid Cen.PK2 and tetrads were dissected. (B) pGal-GPI10 was transformed into RH3699 and tetrads were dissected. Growth on medium containing geneticin was used to monitor the deletion of *GPI10*. (C) p425-PIG-B was transformed into RH3699 and tetrads were dissected onto minimal medium. The growth of two spores (A, B, C and D) is shown: spore A was disrupted for *GPI10*, as monitored by growth on medium containing geneticin, whereas the wild-type spore, B, cannot grow on geneticin-containing plates.

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

Figure 3 Gpi10p depletion causes a GPI-anchoring defect

(A) The growth of the spores of one tetrad [RH3994 (A), RH3995 (B), RH3996 (C), RH3997 (D)] was monitored upon shift from glycerol- to glucose-containing medium. (B) Incorporation of [3 H]myo-inositol into proteins is shown for the spores of the tetrad. Cells were shifted from glycerol- to glucose-containing medium for 16 h. They were then metabolically labelled and chased as indicated. Lysates were prepared which were immunoprecipitated for Gas1p. The signal was detected by exposure and quantification of SDS gels using a PhosphorImager. Quantification of these pulse-chase experiments are shown. The appearance of mature Gas1p indicates GPI-anchor attachment and transport to the Golgi apparatus.

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 [3 H]myo-inositol due to the inositol residue in the GPI anchor. Cells were grown in SGYE 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 [3 H]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 SGYE 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

Figure 4 Gpi10p depletion causes the accumulation of Man₂-GlcN-(acyl)PI and of a novel lipid

pmi40 (RH3998) or *pmi40 gpi10Δ::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. Man₂, Man₂-GlcN-(acyl)PI; MIPC, mannosylinositolphosphorylceramide; M(IP)₂C, mannosyldi-inositolphosphorylceramide; arrow, novel lipid; CP, complete precursor; O, origin.

Figure 3(C), Gas1p maturation was severely delayed in *gpi10Δ::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-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 *gpi10Δ::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 Man₂-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 Man₂-GlcN-(acyl)PI occurred [24]. In Gpi10p-depleted cells, Man₂-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 Man₂-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 Man₂-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-Man₃-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-Man₃-GlcN-PI and Man₃-GlcN-PI [28].

GPI10 overexpression results in partial resistance to YW3548

As YW3548 prevents the addition of the third mannose to the GPI intermediate Man₂-GlcN-(acyl)PI and our data suggest that

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

RH3999 cells were metabolically labelled with [¹⁴C]Man, and total lipids were extracted and analysed by TLC. The bands corresponding to Man₂-GlcN-(acyl)PI, the novel lipid (Figure 4, arrow) and of lipid C [EthN-P-Man₃-GlcN-(acyl)PI] from *Trypanosoma brucei* were eluted from the TLC material by organic solvents. Hydrophilic fragments were generated by nitrous acid deamination and reduction, whereas neutral core glycans were generated by dephosphorylation, deamination and reduction. Hydrophilic fragments and neutral core glycans were analysed by Bio-Gel P4 size-exclusion and Dionex high-pH anion-exchange chromatography (HPAEC) respectively. Partially hydrolysed glucose oligomers were used as internal standards. The elution position of the hydrophilic fragments and the neutral core glycans are indicated as glucose units (GU) and Dionex units (DU) respectively. JBAM, Jack bean α -mannosidase.

Lipid	Bio-Gel P4 Gel filtration analysis of the hydrophilic fragments (GU)	Dionex HPAEC analysis of the neutral core glycans (DU)
EthN-P-Man ₃ -GlcN-(acyl)PI	7.8	2.5
Man ₂ -GlcN-(acyl)PI	3.3	2.1
Novel lipid	7.3	2.1
Novel lipid (JBAM)	7.0/0.9	1.1

Figure 5 *GPI10* overexpression confers partial resistance to the GPI-synthesis inhibitor YW3548

(A) RH1657 cells carrying pGal or pGal-GPI10 were grown in YGal and incubated with 2 μ g/ml YW3548 or methanol and growth was monitored by counting cells in a haemocytometer. (B) RH1657 cells carrying pGal or pGal-GPI10 were included in YGal plates and 4 μ l of different concentrations of YW3548 were spotted on the plates (top, 100 μ g/ml; middle, 10 μ g/ml; and bottom, 1 μ g/ml) before incubation at 30 °C.

GPI10 is required for this step, we decided to test whether overexpression of *GPI10* would lead to resistance to YW3548. To address this question, we transformed wild-type cells with either pGal-GPI10 or the empty pGal control vector. The growth of cells in YGal medium in the presence of YW3548 or methanol as control was monitored (Figure 5A). We observed that cells carrying the control vector were unable to grow in the presence of YW3548, whereas cells that expressed *GPI10* from the *GAL1/10* promoter were able to overcome the effects of the compound and grow, albeit more slowly, than untreated cells. In a different assay, cells carrying either pGal-GPI10 or pGal were included in YGal plates and halo assays were performed (Figure 5B). Cells carrying the control vector gave a completely clear halo upon addition of YW3548. In contrast, overexpression of *GPI10* allowed partial growth in the presence of the compound. Whereas the halo was clear in the beginning, it became turbid after 2 days of incubation. This partial resistance suggests that YW3548 might interfere directly or indirectly with the function of Gpi10p.

DISCUSSION

In this study, we present the characterization of the *GPI10* gene from yeast. A deletion of the essential *GPI10* gene can be complemented by expression of the human *PIG-B* cDNA, suggesting that *GPI10* and *PIG-B* are functional homologues. 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, $\text{Man}_2\text{-GlcN-(acyl)PI}$ as well as a novel lipid were found to accumulate. This lipid contained a $\text{Man}_2\text{-anhydromannitol}$ 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 $\text{Man}_2\text{-anhydromannitol}$. Furthermore, it is sensitive to treatment with Jack bean mannosidase. These findings suggest that the novel lipid may represent a GPI intermediate, most likely $\text{Man-(EthN-P)Man-GlcN-(acyl)PI}$. 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 $\text{Man}_2\text{-GPI}$ intermediate with two mannoses and no ethanolamine accumulated. Here we have observed that the addition of a putative ethanolamine phosphate to the $\text{Man}_2\text{-GPI}$ 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. $\text{Man-(EthN-P)Man-Glc-(acyl)PI}$ could be the natural and highly preferred substrate for Gpi10p, the putative $\alpha 1,2$ mannosyltransferase that adds the third mannose. In this case, the biosynthetic scheme in yeast would be $\text{PI} \rightarrow \text{GlcNAc-PI} \rightarrow \text{GlcN-PI} \rightarrow \text{GlcN-(acyl)PI} \rightarrow \text{Man-GlcN-(acyl)PI} \rightarrow \text{Man-Man-GlcN-(acyl)PI} \rightarrow \text{Man-(EthN-P)Man-GlcN-(acyl)PI} \rightarrow \text{Man-Man-(EthN-P)Man-GlcN-(acyl)PI} \rightarrow \text{Man-Man-Man-(EthN-P)Man-GlcN-(acyl)PI}$ and YW3548 would block the addition of ethanolamine phosphate to the Man_2 intermediate. Consistent with this synthetic scheme we have preliminary data on the structure of a GPI product that accumulates in a mutant that is defective in addition of the mannose side-chain. This product

also appears to have an EthN-P on the first mannose of the core GPI structure (C. Sütterlin, 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 mannose [1]. Previously, we suggested that YW3548 might mimic the $\text{Man}_2\text{-GlcN-(acyl)PI}$ 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 *GP110* 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 that works on the first mannose. In the latter case, Gpi10p may utilize the unmodified $\text{Man}_2\text{-GPI}$ 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 mannose in N-glycan precursor synthesis [36]. The mannose 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 *GP110* 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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REFERENCES

- 1 McConville, M. J. and Ferguson, M. A. J. (1993) *Biochem. J.* **294**, 305–324
- 2 Englund, P. T. (1993) *Annu. Rev. Biochem.* **62**, 121–138
- 3 Takeda, J. and Kinoshita, T. (1995) *Trends Biochem. Sci.* **20**, 367–371

- 4 Udenfriend, S. (1995) *Annu. Rev. Biochem.* **64**, 563–591
- 5 Maxwell, S. E., Ramalingam, S., Gerber, L. D., Brink, L. and Udenfriend, S. (1995) *J. Biol. Chem.* **270**, 19576–19582
- 6 Riezman, H. and Conzelmann, A. (1998) *Handbook for Proteolytic Enzymes* (Barret, A. J., Rawlings, N. D. and Woessner, J. F., eds.), Academic Press, London, in the press
- 7 Nuoffer, C., Horvath, A. and Riezman, H. (1993) *J. Biol. Chem.* **268**, 10558–10563
- 8 Kinoshita, T. and Takeda, J. (1994) *Parasitol. Today* **10**, 139–143
- 9 Stevens, V. L., Yhang, H. and Harrenman, M. (1996) *Biochem. J.* **313**, 253–258
- 10 Mohny, R. P., Knez, J. J., Ravi, L., Seveler, d., Rosenberry, T. L., Hirose, S. and Medof, M. E. (1994) *J. Biol. Chem.* **269**, 6536–6542
- 11 Nakamura, N., Inoue, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V. L. and Kinoshita, T. (1997) *J. Biol. Chem.* **272**, 15834–15840
- 12 Sugiyama, E., DeGasperi, R., Urakaze, M., Chang, M., Thomas, L. J., Hyman, R., Warren, C. D. and Yeh, E. T. H. (1991) *J. Biol. Chem.* **266**, 12119–12122
- 13 Takahashi, M., Inoue, N., Ohishi, K., Maeda, Y., Nakamura, N., Endo, Y., Takeda, J. and Kinoshita, T. (1996) *EMBO J.* **15**, 4254–4261
- 14 Miyata, T., Takeda, J., Iida, Y., Yamada, N., Inoue, N., Takahashi, M., Maeda, K., Kitani, T. and Kinoshita, T. (1993) *Science* **259**, 1318–1320
- 15 Inoue, I., Watanabe, R., Takeda, J. and Kinoshita, T. (1996) *Biochem. Biophys. Res. Commun.* **226**, 193–199
- 16 Inoue, N., Kinoshita, T., Orii, T. and Takeda, J. (1993) *J. Biol. Chem.* **268**, 6882–6885
- 17 Kamitani, T., Chang, H., Rollins, C., Waneck, G. L. and Yeh, E. T. H. (1993) *J. Biol. Chem.* **268**, 20733–20736
- 18 Leidich, S. D., Kostova, Z., Latek, R. R., Costello, L. C., Drapp, D. A., Gray, W., Fassler, J. S. and Orlean, P. (1995) *J. Biol. Chem.* **270**, 13029–13035
- 19 Schönbächler, M., Horvath, A., Fassler, J. and Riezman, H. (1995) *EMBO J.* **14**, 1637–1645
- 20 Hamburger, D., Egerton, M. and Riezman, H. (1995) *J. Cell Biol.* **129**, 629–639
- 21 Benghezal, M., Benachour, A., Rusconi, S., Aebi, M. and Conzelmann, A. (1996) *EMBO J.* **15**, 6575–6583
- 22 Benghezal, M., Lipke, P. N. and Conzelmann, A. (1995) *J. Cell Biol.* **130**, 1333–1344
- 23 Menon, A. K., Mayor, S. and Schwarz, R. T. (1990) *EMBO J.* **9**, 4249–4258
- 24 Sütterlin, C., Horvath, A., Gerold, P., Schwarz, R. T., Wang, Y., Dreyfuss, M. and Riezman, H. (1997) *EMBO J.* **16**, 6374–6383
- 25 Wach, A., Brachat, A., Pöhlmann, R. and Philippsen, P. (1994) *Yeast* **10**, 1793–1808
- 26 Horvath, A., Sütterlin, C., Manning-Krieg, U., Movva, N. R. and Riezman, H. (1994) *EMBO J.* **13**, 3687–3695
- 27 Sipos, G., Puoti, A. and Conzelmann, A. (1994) *EMBO J.* **13**, 2789–2796
- 28 Gerold, P., Striepen, B., Reitter, B., Geyer, H., Geyer, R., Reinwald, E., Risse, H.-J. and Schwarz, R. T. (1996) *J. Mol. Biol.* **261**, 181–194
- 29 Ferguson, M. A. J. (1991) *Lipid Modification of Proteins: A Practical Approach* (Hooper, N. M. and Turner, A. J., eds.), IRL Press, Oxford
- 30 Nuoffer, C., Jenö, P., Conzelmann, A. and Riezman, H. (1991) *Mol. Cell. Biol.* **11**, 27–37
- 31 Doering, T. L. and Schekman, R. (1996) *EMBO J.* **15**, 182–191
- 32 Puoti, A., Desponds, C., Fankhauser, C. and Conzelmann, A. (1991) *J. Biol. Chem.* **266**, 21051–21059
- 33 DeGasperi, R., Thomas, L. J., Sugiyama, E., Chang, H. M., Beck, P. J., Orlean, P., Albright, C., Sambrook, J. F., Warren, C. D. and Yeh, E. T. H. (1990) *Science* **250**, 988–991
- 34 Leidich, S. D., Drapp, D. A. and Orlean, P. (1994) *J. Biol. Chem.* **269**, 10193–10196
- 35 Fankhauser, C., Homans, S. M., Thomas-Oates, J. E., McConville, M. J., Desponds, C., Conzelmann, A. and Ferguson, M. A. J. (1993) *J. Biol. Chem.* **268**, 26365–26374
- 36 Burda, P., te Heesen, S., Brachat, A., Wach, A., Dusterhof, A. and Aebi, M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7160–7165