GPI anchor biosynthesis in yeast: phosphoethanolamine is attached to the α1,4-linked mannose of the complete precursor glycophospholipid

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Cells synthesize the GPI anchor carbohydrate core by successively adding N-acetylglucosamine, three mannoses, and phosphoethanolamine (EtN-P) onto phosphatidylinositol, thus forming the complete GPI precursor lipid which is then added to proteins. Previously, we isolated a GPI deficient yeast mutant accumulating a GPI intermediate containing only two mannoses, suggesting that it has difficulty in adding the third, α1,2-linked Man of GPI anchors. The mutant thus displays a similar phenotype as the mammalian mutant cell line S1A-b having a mutation in the PIG-B gene. The yeast mutant, herein named *gpi10–1*, contains a mutation in YGL142C, a yeast homolog of the human PIG-B. YGL142C predicts a highly hydrophobic integral membrane protein which by sequence is related to *ALG9*, a yeast gene required for adding Man in α1,2 linkage to N-glycans. Whereas *gpi10–1* cells grow at a normal rate and make normal amounts of GPI proteins, the microsomes of *gpi10–1* are completely unable to add the third Man in an *in vitro* assay. Further analysis of the GPI intermediate accumulating in *gpi10* shows it to have the structure Manα1–6(EtN-P-)Manα1–4GlcNα1–6(acyl)Inositol-P-lipid. The presence of EtN-P on the α1,4-linked Man of GPI anchors is typical of mammalian and a few other organisms but had not been observed in yeast GPI proteins. This additional EtN-P is not only found in the abnormal GPI intermediate of *gpi10–1* but is equally present on the complete GPI precursor lipid of wild type cells. Thus, GPI biosynthesis in yeast and mammals proceeds similarly and differs from the pathway described for *Trypanosoma brucei* in several aspects.

Key words: ethanolamine/glycosylphosphatidylinositol/membrane anchors/N-glycosylation/*Saccharomyces cerevisiae*

Introduction

Many glycoproteins of lower and higher eukaryotes are attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI) (Englund, 1993; McConville and Ferguson, 1993). Where investigated, the GPI anchoring pathway has been found to be essential. In man, somatic mutations in the GPI anchoring pathway of hematopoietic stem cells cause a hemolytic disease called paroxysmal nocturnal hemoglobinuria. Similarly, targeted deletion of the GPI pathway in the mouse model shows that the pathway is of vital importance for fetal and neonatal development (Dunn *et al.*, 1996; Kawagoe *et al.*, 1996; Tarutani *et al.*, 1997). GPI anchoring also is essential for *Saccharomyces cerevisiae* (Leidich *et al.*, 1994, 1995; Hamburger *et al.*, 1995; Schönbächler *et al.*, 1995; Vossen *et al.*, 1995) since many cell wall proteins are primarily anchored via a GPI (de-Nobel and Lipke, 1994; Van-der-Vaart *et al.*, 1997).

GPI proteins have a classical signal sequence at their N-terminus and a GPI anchoring signal at their C-terminus, which latter, after import of the protein into the ER, is removed and replaced by a preformed GPI (Caras *et al.*, 1987; Moran and Caras, 1991; Moran *et al.*, 1991; Kodukula *et al.*, 1993).

GPI anchor biosynthesis seems to have been elaborated early in evolution since the oligosaccharide core linking the protein to the lipid moiety has the same structure in all organisms analyzed so far, namely protein-CO-NH-$(CH_2)_2$-PO_4–6Manα1–2Manα1–6Manα1–4GlcNH_2-*myo*-inositol-PO_4-lipid. Apart from this carbohydrate core, GPI anchors from various species differ widely by the kind of side chains attached to the core structure and by their lipid moieties (McConville and Ferguson, 1993).

One characteristic side chain described in mammals and also in *Torpedo californica* consists of a phosphoethanolamine (EtN-P) attached to the C2 position of the α1,4-linked Man (Man1, Figure 1). As for many other side chains, no functional role has been attributed yet to this modification. The previous structural analysis of mature yeast GPI proteins did not reveal the presence of this EtN-P residue (Fankhauser *et al.*, 1993). However, while analyzing the structure of an abnormal glycolipid accumulating in mutant 839 (Benghezal *et al.*, 1995) we realized that it carried an EtN-P and has the structure Manα1–6(EtN-P-)Manα1–4GlcNα1–6(acyl)Ins-P-lipid. This finding was quite unexpected and triggered further investigations which revealed that the EtN-P substituent on Man1 was also present on the complete GPI precursor lipid (CP2) ready to be transferred to proteins, whereas the incomplete GPI intermediate of mutant 839 could not be linked up to proteins.

We also report on the further characterization of mutant 839 and show that its GPI deficiency is due to a mutation in YGL142C, a predicted open reading frame of *S.cerevisiae* which has homology to PIG-B (Takahashi *et al.*, 1996). PIG-B is a human gene which, if mutated, also causes the accumulation of the same Manα1–6(EtN-P-)Manα1–4GlcNα1–6(acyl)Ins-P-lipid intermediate (Puoti *et al.*, 1991; Takahashi *et al.*, 1996).

Results

M2 accumulation of strain 839 segregates as a Mendelian recessive trait and can be corrected by YGL142C*

We recently reported on the isolation of yeast mutants deficient in the biosynthesis of GPI anchors (Benghezal *et al.*, 1995). Mutant 839 accumulated M2*, an abnormal, incomplete GPI

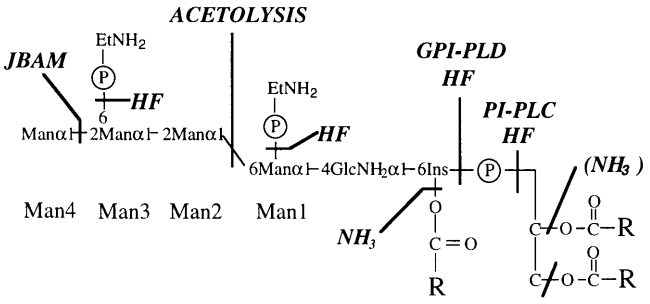

Fig. 1. Presumed structure of the complete yeast precursor glycolipid CP2**.** Relevant cleavage procedures are indicated. Man1, Man2, Man3, and Man4 will be used in the text to designate the α1,4-linked, α1,6-linked, and α1,2-linked Man, respectively.

containing a Man_2-GlcN-Ins carbohydrate core but still incorporated some [^{3}H]Ins into proteins and thus was still able to make GPI proteins at 37°C. 839 was sterile but could be out crossed to X2180–1A by spheroplast fusion, and M2* accumulating spores were then back crossed twice to W303. In the last back cross the M2* accumulation segregated in a 2:2 manner in 4 out of 4 tetrads and was no more associated with sterility nor with the slight *ts* growth phenotype of the original mutant. M2* accumulating strains were named *gpi10–1*. After crossing the *gpi10–1* mutants incorporated perfectly normal amounts of [^{3}H]Ins into proteins. We could suppress M2* accumulation by transfecting a multicopy plasmid with YGL142C, a yeast open reading frame which is homologous to the human PIG-B gene (30.5% identity, 57.4% similarity) as shown in Figure 2, lane 2. When the URA3-based YGL142C-containing plasmid was forced out again with 5 fluoroorotic acid, M2* accumulation reappeared (Figure 2, lane 3). YGL142C on a single copy vector greatly reduced M2* accumulation (Figure 2, lane 4). When the gene was integrated into the genome, the accumulation of this intermediate was no more observed (lane 5). Sequencing of YGL142C and its flanking regions in *gpi10–1* revealed the presence of a single point mutation consisting of a G to A transition which changes Ala48 into Thr. Ethylmethanesulfonate, by which the mutant was generated, almost exclusively produces G · C to A · T transitions (Kohalmi and Kunz, 1988).

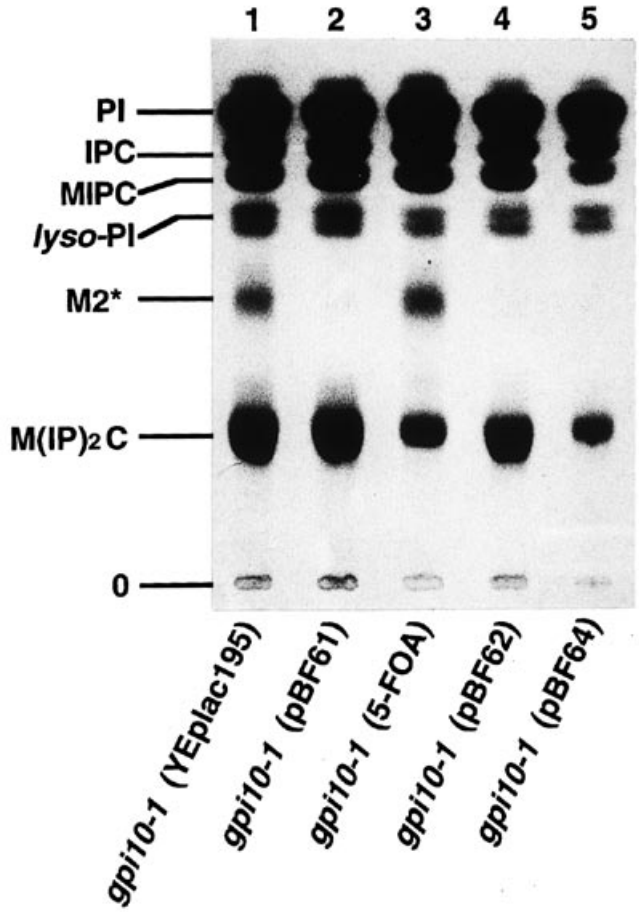

Fig. 2. Complementation of *gpi10–1* by YGL142C**.** 2.5 OD_{600} of FBY169 cells containing different plasmids were labeled at 37°C in SDCA medium using 2 μCi [2–^{3}H]Ins/OD_{600} unit of cells. The lipids were extracted and analyzed by TLC using solvent 2. 5-FOA, 5 fluoroorotic acid was used to select against plasmid pBF61 of the strain shown in lane 2. pBF64 has no ARS sequence and can only be inherited after integration into the genome. O, Origin.

Microsomes of wild type cells make complete GPIs in vitro

To further characterize the defect of 839 we used a previously described microsomal *in vitro* system (Costello and Orlean, 1992) transferring [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc to PI, thus generating [^{3}H]GlcNAc-PI, [^{3}H]GlcN-PI and [^{3}H]GlcN-(acyl)PI. As reported previously (Costello and Orlean, 1992), the addition of ATP and Coenzyme A greatly enhanced the production of [^{3}H]GlcN-(acyl)PI (not shown). In the presence of GDP-Man wild type microsomes made several additional lipid species (Figure 3A), the most polar of which (lipid k) comigrated with the complete GPI precursor CP2 (Figure 3A, lanes 2, 3). No additional bands were observed when tunicamycin was omitted and the addition of the chitin synthase inhibitor nikkomycin did not increase the incorporation of [^{3}H]GlcNAc into lipids (not shown). All lipids made by the microsomes were purified by two rounds of preparative TLC, their head groups were dephosphorylated by HF, N-acetylated, desalted and finally analyzed by TLC as described previously (Sipos *et al.*, 1994). This allowed to define the number of Man residues attached to the GlcN-Ins core of these intermediates (Figure 3 caption). Lipids h, i, j, and k contained four Man residues. We sought further confirmation that lipid k was indeed identical with CP2, i.e., the most polar complete GPI precursor generated *in vivo* (Sipos *et al.*, 1994). For this the lipid extract of labeled microsomes was treated with PI-PLC and with jack bean α-mannosidase (JBAM). Lipid k was found to be resistant to PI-PLC whereas the nonacylated GlcNAc-PI and GlcN-PI were not (Figure 3A, lane 3). Lipid k also was transformed into a lipid with a slightly higher R_f by JBAM (lane 4) and was quantitatively cleaved by GPI-PLD (not shown). Thus, the partial structural analysis shows that lipid k behaves in all respects as the authentic CP2 of intact cells (Sipos *et al.*, 1994). Since the microsomal system used here does not support vesicular traffic, the result demonstrates, that all the enzymes required to make CP2 are contained in the ER.

Biosynthesis of complete GPI precursors was previously observed to be dependent on the addition of GDP-Man in the analogous system from *T.brucei* (Masterson *et al.*, 1989; Menon *et al.*, 1990). It thus appears that in both yeast and trypanosomal microsomes the obligatory intermediate Dol-P-Man is limiting but can be replenished by the addition of GDP-Man.

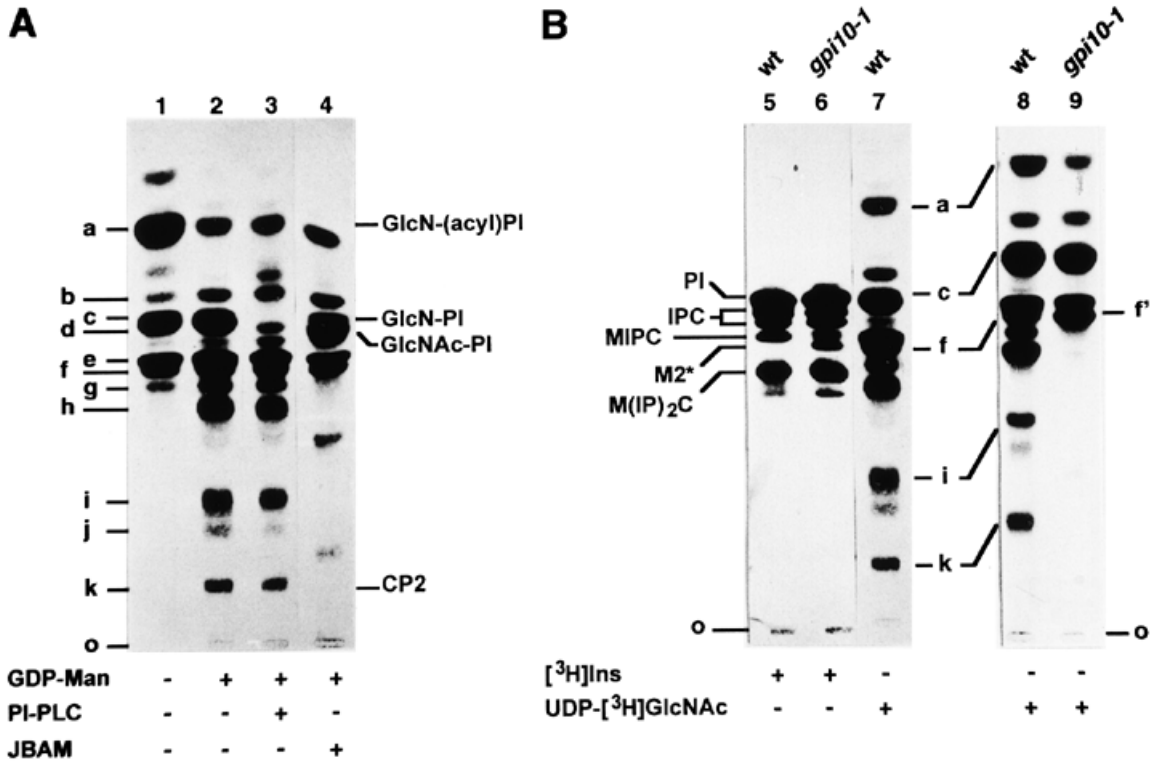

Fig. 3. Incorporation of UDP-[^{3}H]GlcNAc into GPIs by microsomes *in vitro*. (**A**) Microsomes of W303 wild type cells were incubated with 6 μCi of UDP-[^{3}H]GlcNAc, tunicamycin, nikkomycin and ATP, in presence (+) or absence (-) of GDP-Man for 1 h at 37°C. The glycolipid products were extracted and separated by TLC. (**B**) W303 wild type and 839 cells were labeled with [^{3}H]Ins (lanes 5, 6) and their microsomes were labeled with UDP-[^{3}H]GlcNAc in the presence of GDP-Man (lanes 7–9). The number of Man residues attached to the GlcN-Ins core of lipids a–k was found to be 0 for a, c and d; 1 for b and e; 4 for h, i, j, and k. Bands f and g contained mixtures: 0 or 1 Man in band f, 1 or 2 Man in band g. The most polar UDP-[^{3}H]GlcNAc-labeled lipid of 839 comigrating with f is labeled f′.

Microsomes of 839 are unable to make complete GPIs in vitro

For further characterization of the mutation in 839, we assayed the ability of its microsomes to synthesize GPI precursor lipids *in vitro*. Under conditions in which wild type microsomes made CP2, mutant microsomes made abnormally high amounts of a less polar species (Figure 3B, lane 9, band f′) but none of the more polar species. Lipid f′ comigrated with the [^{3}H]Ins labeled abnormal GPI lipid M2* accumulating *in vivo* which had originally led to the isolation of this mutant (Benghezal *et al.*, 1995) (Figure 3B). Further characterization of lipid f′ was required since HF dephosphorylation showed that the corresponding lipid f made by wild type microsomes contained intermediates with 0 or 1 Man only, whereas the *in vivo* made M2* of 839 had previously been shown to contain 2 Man residues (Benghezal *et al.*, 1995). Therefore, the abnormal GPI lipids of 839 generated *in vivo* (M2*) or *in vitro* (f′) were isolated by two consecutive rounds of preparative TLC and soluble head groups were obtained using methanolic ammonia followed by PI-PLC as described (Sipos *et al.*, 1994). The head groups were then fragmented by HF dephosphorylation and acetolysis as shown in Figure 4. In addition to the M0 fragment obtained from lipid f made by wild type microsomes, HF treatment of lipid f′ of 839 yielded an additional major fragment containing two Man (Figure 4A, lane 1). The same fragment was obtained from M2* made *in vivo* in addition to Ins stemming from a major [^{3}H]Ins-labeled contaminant having copurified with M2*, probably *lyso*-PI (Figure 4B, lane 1′). The result indicates that microsomes from 839 indeed accumulate the same abnormal GPI intermediate as the intact mutant cell. Further analysis of f′ and M2* using combinations of HF dephosphorylation and acetolysis confirmed that one of the two Man of this intermediate was linked α1,6 (Figure 4, lanes 3, 5, 3′, 5′). Experiments with JBAM showed that f′ and M2* contained an HF sensitive substituent on Man1 as shown in Figure 4: JBAM done before HF (lanes 4, 4′) yielded a Man_1-GlcN-Ins fragment (M1) whereas JBAM after HF yielded GlcN-Ins (M0) fragment (lanes 2, 2′). The treatment of head groups in the order acetolysis, then JBAM, then HF (lanes 6, 6′) showed that after removal of the α1,6-linked Man, the protected Man was still present. We conclude that it is not the α1,6-linked Man that carries the HF sensitive substituent. The presence of a substituent on Man1 could be confirmed by a JBAM treatment of the intact [^{3}H]Ins-labeled M2* lipid from 839 cells. JBAM treatment shifted the purified M2* to a slightly less polar position but didn't generate the GlcN-(acyl)PI intermediate (M0) expected for a PI-PLC-resistant GPI intermediate having two Man but no other substituents (Figure 5, lane 2). The heterogeneity of purified M2* is analogous to the one observed with purified GlcN-(acyl)PI and is probably due to migration of the acyl chain on the *myo*-inositol (Sipos *et al.*, 1997).

Two types of HF-sensitive substituents on Man1 have been described in the literature so far. Mammalian GPI proteins invariably have been found to contain an additional EtN-P group on the conserved carbohydrate core and in several cases the linkage of this group has been determined and found to attach EtN-P by a phosphodiester bond to the C2 of Man1 (Tse *et al.*,

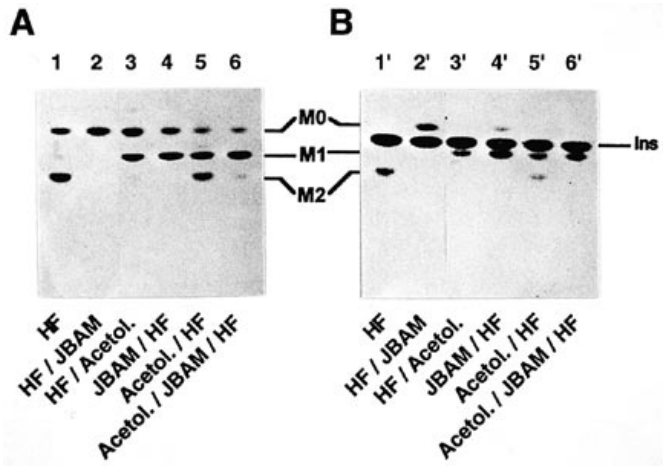

Fig. 4. Structural analysis of head groups of lipid f' and M2*. (**A**) Microsomes of 839 were labeled with UDP-[^{3}H]GlcNAc, lipid f′ was purified by preparative TLC and its head group was obtained. (**B**) 839 cells were metabolically labeled with [^{3}H]Ins; lipid M2* was purified, and its head group was obtained as above. Acetolysis (Acetol.), JBAM, and HF treatments were carried out in the order indicated at the bottom of each lane. After these treatments, all samples were N-acetylated, desalted, and analyzed by TLC. M0, GlcNAc-Ins; M1, Man$_1$-GlcNAc-Ins; M2, Man$_2$-GlcNAc-Ins. Lipid f′ was contaminated with a lipid species giving rise to a M0 HF fragment (lane 1). M2* contained a major contaminant yielding Ins (probably lyso-PI). Note that acetolysis is not always quantitative in conditions in which only α1,6-glycosidic bonds are to be cleaved (Schneider *et al.*, 1990).

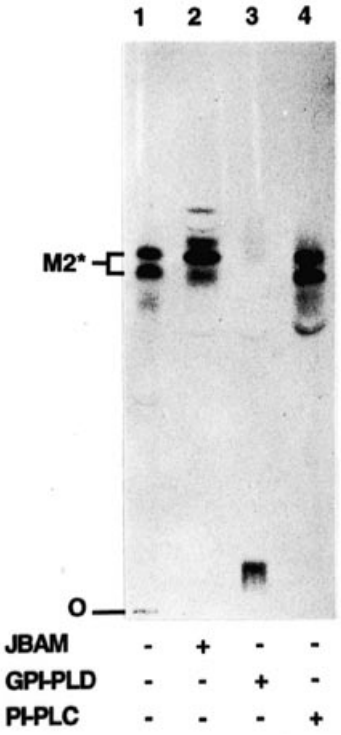

Fig. 5. Analysis of the M2* glycolipid of *gpi10*. [^{3}H]Ins-labeled M2* from 839 was purified as in Figure 4. After PI-PLC (lane 4), GPI-PLD (lane 3), and JBAM (lane 2) the lipids were separated by TLC in solvent 1. A nontreated control is shown in lane 1.

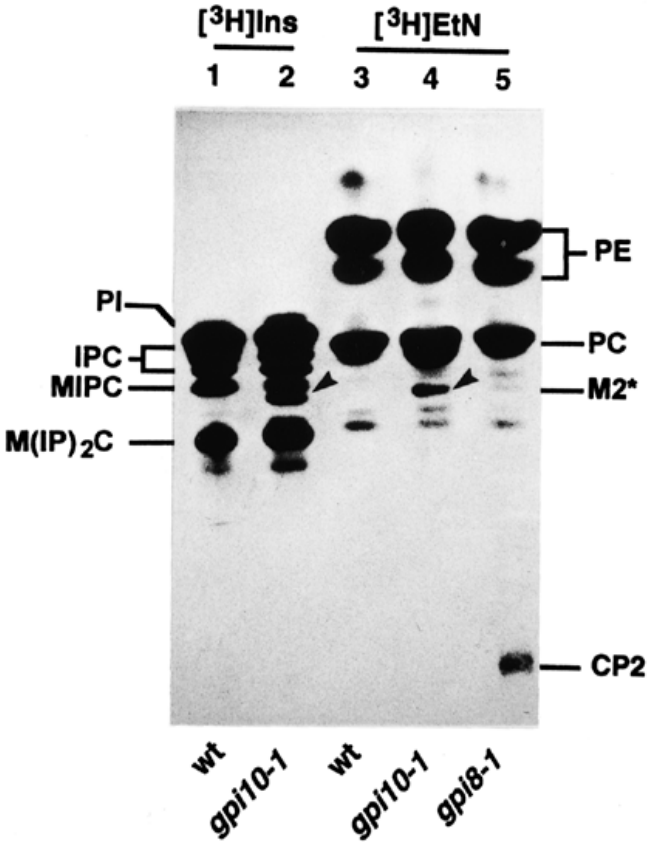

Fig. 6. Lipids from yeast strains metabolically labeled with [^{3}H]EtN. W303 wild type (wt), FBY11 (*gpi8–1*), and FBY169 (*gpi10–1*) were metabolically labeled with [^{3}H]EtN or [^{3}H]Ins at 37°C. [^{3}H]Ins-labeled (lanes 1, 2) or [^{3}H]EtN-labeled lipids (lanes 3, 4, 5) were separated by TLC with solvent 1. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were the main [^{3}H]EtN-labeled species. Arrowheads point to the abnormal lipids of *gpi10–1* cells.

1985; Homans *et al.*, 1988; Roberts *et al.*, 1988b; Walter *et al.*, 1990; Stahl *et al.*, 1992; Redman *et al.*, 1994; Taguchi *et al.*, 1994; Treumann *et al.*, 1995). An additional EtN-P has also been found in lower vertebrates (Mehlert *et al.*, 1993) and in *Dictyostelium discoideum* (Haynes *et al.*, 1993). EtN-P on Man1 was found to be present already at the level of the mammalian precursor glycolipids (Hirose *et al.*, 1992; Kamitani *et al.*, 1992; Puoti and Conzelmann, 1992; Ueda *et al.*, 1993).

The other type of HF-sensitive substituent was found in the GPI precursor glycolipids of *Paramecium primaurelia* and consists of Man, again linked via a phosphodiester to Man1 (Azzouz *et al.*, 1995).

In an attempt to elucidate the identity of the HF sensitive substituent on Man1, we compared the lipid extract of [^{3}H]EtN-labeled 839 and wild type cells and found that 839 contains an abnormal, [^{3}H]EtN-labeled lipid in the same region as M2*, which is not seen in wild type cells nor in CP2 accumulating *gpi8* cells (Figure 6). This [^{3}H]EtN-labeled lipid of 839 was sensitive to GPI-PLD (not shown). These results strongly suggest that the additional substituent on Man1 consists of EtN-P and that M2* and lipid f′ accumulating in 839 have the structure Manα1–6(EtN-P-)Manα1–4GlcNα1–6(acyl)Inositol-P-lipid.

GPI anchors of 839 contain four mannoses

Results showed that 839 incorporates [^{3}H]Ins into proteins and is able to make GPI anchored proteins in spite of the fact that its microsomes are completely unable to add Man3. It therefore

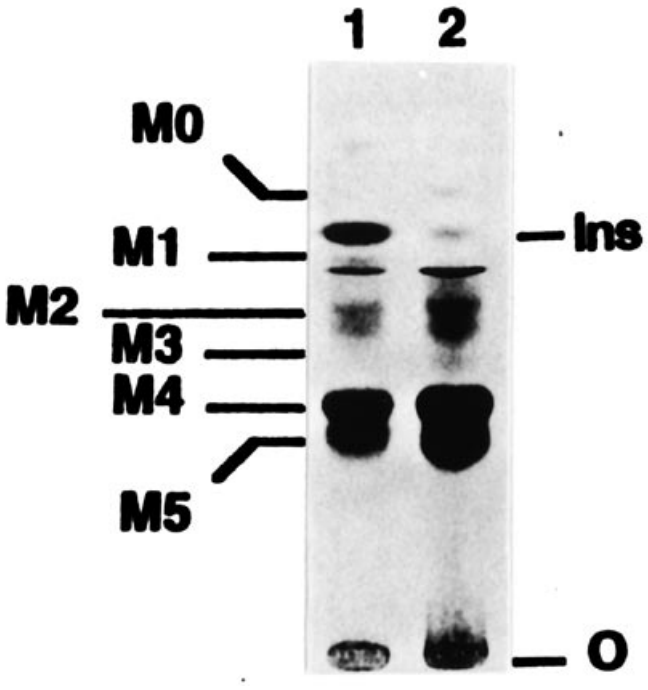

Fig. 7. Analysis of the GPI-anchor structure from W303 (lane 1) and *gpi10–1* (lane 2). Cells were labeled with [^{3}H]Ins and GPI-anchored peptides were obtained as described in *Materials and methods*. Anchor peptides were subjected to HF treatment, N-acetylated, desalted, and separated by TLC. M0 to M5, Man_0-GlcNAc-Ins to Man_5-GlcNAc-Ins.

seemed to be a possibility that the EtN-P on Man1 may serve as an alternative attachment point for the GPI protein. To test for this, we labeled 839 and wild type cells with [^{3}H]Ins, prepared anchor peptides from the labeled GPI proteins and treated them with HF. As can be seen on Figure 7, 839 contains the same M4 and M5 fragments as wild type cells. A minor M2 fragment is present in both, wild type and 839 cells but has not been observed in previous experiments of the same type in wild type cells (Sipos *et al.*, 1995). The minor M2 fragment probably is due to some non specific hydrolysis of glycosidic bonds during HF treatment in this experiment.

We conclude that 839, in spite of the complete biosynthetic block observed *in vitro*, is able to make complete anchor precursor lipids *in vivo*. The GPI transamidase transferring the GPI onto precursor proteins does not seem to be able to use the accumulating M2* glycolipid as an alternative anchoring device, at least not in a situation in which complete precursor lipids are also available.

An HF sensitive substituent is also present on the α1,4-linked Man of the complete GPI precursors

The presence of EtN on Man1 came as a surprise to us since previous structural analysis of the GPI anchor of yeast proteins had not unveiled any EtN-P residue on Man1 (Fankhauser *et al.*, 1993), although this analysis relied on methods which had detected such a residue on the mammalian GPI anchor (Homans *et al.*, 1988). We therefore asked, if the addition of EtN-P to Man1 is a physiological event or if it only occurs under special circumstances when an abnormal intermediate is accumulating, as is the case in 839. For this we reinvestigated the structure of the complete GPI precursors CP1 and CP2 of *pmi40*, a mutant with a conditional defect (*ts*) in Man biosynthesis allowing for efficient metabolic labeling of otherwise undetectable GPI lipids (Sipos *et al.*, 1994). At the same time we also analyzed M4*, a minor, less polar GPI lipid of *pmi40* which comigrates with the major abnormal lipid accumulating in *gpi7* (Benghezal *et al.*, 1995) (Figure 8, top). To assess the presence of an HF sensitive substituent on Man1, the head groups of [^{3}H]Man-labeled CPs were first cleaved by acetolysis, a procedure which in mild conditions specifically cleaves α1,6-glycosidic bonds (Figure 1). Here this procedure is expected to produce the two fragments Manα1,2(EtN-P-)Manα1,2Man and (X?-)Manα1,4GlcN-α1,6Ins with X being the hypothetical substituent in question (Figure 1). The fragments were then treated with JBAM or control incubated and finally dephosphorylated by HF. The resulting fragments were analyzed by paper chromatography. As can be seen in Figure 8, the (X?-)Manα1,4GlcNα1,6Ins of CPs is JBAM resistant since after HF we obtain Manα1,4GlcN-α1,6Ins (M1) but the analogous fragment of M4* is JBAM sensitive. Since JBAM treatment after HF reduces all fragments to free Man and GlcN-Ins (not shown; Sipos *et al.*, 1994), the result clearly indicates that CPs contain an HF sensitive substituent on Man1 which is not present on M4*. On the other hand both CPs and M4* seem to contain an HF sensitive group on Man3 since the residual M4 fragments which survived acetolysis were degraded by JBAM to M3 not to M1 or M0 (Figure 8). The same conclusion can be reached by looking at the Man4-Man3-Man2 (M-M-M) fragments generated by acetolysis: they are not completely degraded by JBAM but are shifted to a smaller fragment (M-M). From the presence of EtN-P on Man1 of M2* of 839, we infer that X on Man1 of CP2 most likely is also an EtN-P.

Analysis of the dolichol-linked glycans

The biosynthesis of the Dol-PP-$GlcNAc_2$-Man_9-Glc_3 precursor for N-glycosylation involves the addition of four different α1,2-linked Mans, two of which are added on the cytosolic side from GDP-Man the other two being added on the lumenal side of the ER from Dol-P-Man (Abeijon and Hirschberg, 1992). *ALG9* encodes a putative α1,2-mannosyltransferase transferring Man from Dol-P-Man to the Dol-PP-$GlcNAc_2$-Man_6 intermediate on the lumenal side of the ER (Burda *et al.*, 1996). This transfer reaction is similar to the transfer of the Man3 in GPI biosynthesis which presumably is operated by YGL142C: in both reactions Man is transferred from Dol-P-Man and is added in α1,2 linkage onto Man. YGL142C indeed shows a low degree of homology to *ALG9* (22.5% identity and 49.5% similarity over 555 amino acids). Since the second lumenal ER α1,2-mannosyltransferase for the synthesis of N-glycans is not yet identified, we analyzed the profile of dolichol-linked oligosaccharides in *gpi10–1*. The mutant showed a perfectly normal profile and in particular, there was no accumulation of Dol-PP-$GlcNAc_2$-Man_7 or Dol-PP-$GlcNAc_2$-Man_8 (data not shown). Thus, there is no indication that YGL142C is involved in the addition of α1,2-linked Man during N-glycan biosynthesis.

Discussion

The GPI core structure linking the protein to the lipid moiety is identical amongst all eukaryotes, but the biosynthetic pathway for its elaboration evolved in slightly different ways in different organisms. The differences are of practical interest in that they may allow to develop drugs against pathogenic protozoa such as trypanosomes, leishmania, and malaria, protozoans in which the

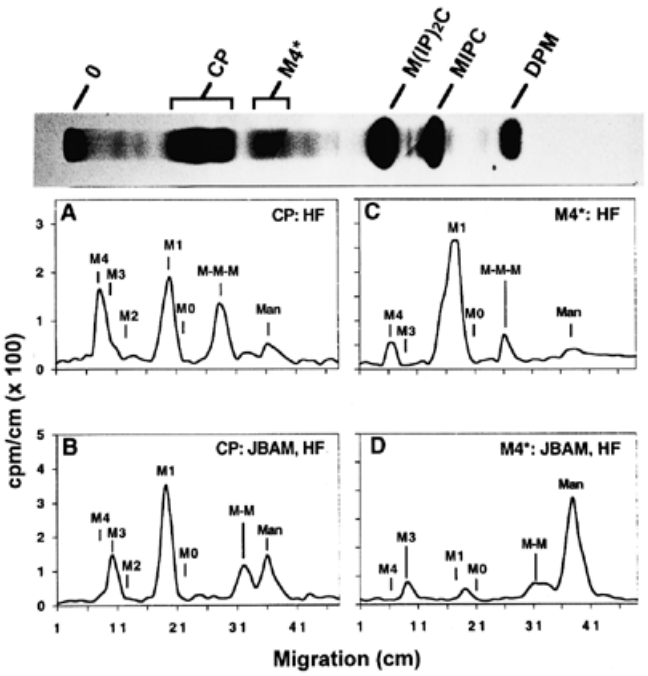

Fig. 8. Fragmentation of CPs (**A**, **B**) and M4* (**C**, **D**) using acetolysis, JBAM and HF. [^{3}H]Man-labeled GPI lipids from *pmi40* cells were purified by preparative TLC. CP1 and CP2 having the same head group (Sipos *et al.*, 1994) were pooled (CP), and similarly, a broad region containing lipids with 4 Man was united (M4*) as indicated at the top. Head groups were liberated, subjected to acetolysis, and further treated as follows: (**A**, **C**) HF; (**B**, **D**) JBAM, then HF. All fragments were N-acetylated, desalted and separated by paper chromatography. The paper was cut into 1 cm wide strips, their radioactivity was determined through scintillation counting. The position of standards run in parallel on the same paper are indicated: M1 to M4, Man_1- to Man_4-GlcNAc-Ins; M-M, Man3-Man2; and M-M-M, Man4-Man3-Man2. DPM, Dolicholphosphomannose. It may be striking that the M-M-M fragments are not prominent than the M1 fragments although they contain three times more Man. However, we noted that the peracetylated Man4-(EtN-P-)Man3-Man2, due to the presence of the hydrophilic EtN-P group, is not efficiently extracted into chloroform after acetolysis and thus gets partially lost during this procedure.

GPI proteins are highly abundant (McConville and Ferguson, 1993). One difference concerns the attachment of a fatty acid to the *myo*-inositol, an event which seems to occur at a different stage of biosynthesis in trypanosomes than in mammalian cells (Güther and Ferguson, 1995; Doerrler *et al.*, 1996). Another notable variation is observed with respect to the EtN-P side chain on Man1 which seems to be invariably present in mammalian GPI proteins, but which is not added in *T.brucei*, nor *L.major*, nor *Plasmodium falciparum* (McConville and Ferguson, 1993; Gerold *et al.*, 1994). Here we document the existence of an EtN-P on Man1 of M2* as well as of CPs of yeast, i.e., the complete precursor lipid. In contrast to other organisms, yeast does not make GPIs in excess of what is needed for the anchoring of proteins and the pool size of complete GPIs is extremely low. The paucity of material only allowed for a partial structural analysis of the [^{3}H]Man-labeled CPs from *pmi40* cells (Sipos *et al.*, 1994). During this previous analysis we therefore did not look for an EtN-P side chain since the extensive structural analysis of the anchor peptides from mature GPI proteins of yeast had not revealed the presence of such an EtN-P side chain on Man1 (Fankhauser *et al.*, 1993).

Here we present evidence for the existence of several GPI precursor lipids containing the Man_4-GlcN-Ins core. Microsomes generated four such intermediates (Figure 3A, bands h–k), and the reinvestigation of the [^{3}H]Man-labeled GPI lipids of *pmi40* made *in vivo* reveals two intermediates, M4* and CPs both having an HF sensitive group on M3. The presence of EtN-P on Man1 of CPs but not M4* offers an explanation for the different mobility of these two compounds in TLC.

The presence of EtN-P on Man1 opens the opportunity to study the enzymes involved in the attachment of this residue in *S.cerevisiae* and thus to obtain information which potentially is relevant for the mammalian system. The mammalian Thy1-deficient mutant cell line S1A-b being deficient in the PIG-B gene (Takahashi *et al.*, 1996) as well as *gpi10–1* accumulate an intermediate having the head group Manα1–6(EtN-P-)Manα1–4GlcN1–6Ins (Puoti *et al.*, 1991; Hirose *et al.*, 1992) indicating that EtN-P can be added to Man-1 before addition of Man3 in both organisms. Thus, the similarity of the yeast with the mammalian system may also extend to the order in which the anchor components are added. It may be noteworthy that the microsomal yeast *in vitro* system made several GPI intermediates containing only 1 Man; nevertheless, digestion of the microsomal lipid extract with JBAM did not lead to an increase of GlcN-acyl-PI (M0) suggesting that the single Man in these intermediates may already be protected by an EtN-P. Addition of EtN-P onto Man1-GlcN-acylPI has also been claimed to occur in mammalian cells (Hirose *et al.*, 1992). In spite of the several analogies between the yeast and the mammalian systems, there seems to be a major discrepancy in that the EtN-P on Man1 is not found on mature yeast GPI proteins although it is invariably present in mammalian GPI proteins. The following possibilities can be considered. (1) The previous analysis of yeast proteins (Fankhauser *et al.*, 1993) may have missed the EtN-P on Man1, since the preparation of GPI anchor peptides in that study was a relatively lengthy procedure during which some hydrolysis of anchor components may have occurred. (2) M4* may be the only GPI which is transferred onto proteins. In this case, CPs may serve some other, yet unknown purpose. (3) CPs may be transferred to proteins but the EtN-P may be removed from Man1 soon after transfer. EtN-P would therefore play a transient role during biosynthesis and attachment of GPIs. The fatty acid attached to the Ins of GPI anchors is a precedent for a component with a transient function in GPI anchor biosynthesis. Preliminary data indicate that an HF-sensitive group indeed can be found on Man1 of certain GPI proteins. GPIs are not only anchoring devices but they are recognized physiologically by numerous proteins and enzymes. They are cleaved by GPI-specific phospholipases, they represent high affinity binding sites for bacterial toxins such as aerolysins and pertussis toxin as well as for interleukin-1β and, at least in yeast, they are substrates for an extracellular transglycosidation reaction, by which GPI proteins get covalently attached to the β1,6-glycans of the cell wall (Davitz *et al.*, 1989; Fukushima *et al.*, 1997; Kollar *et al.*, 1997; Nelson *et al.*, 1997; Rossjohn *et al.*, 1997). It will be of interest to study the influence of the EtN-P side chain on Man1 on these events.

Comparison of YGL142C with its human homolog PIG-B shows 30.5% identity and 57.4% conserved changes. As PIG-B, YGL142C predicts a very hydrophobic protein with multiple transmembrane domains. Apart from *ALG9* there are two additional open reading frames encoding for highly hydrophobic proteins with 22.5% (YOR149C) and 20.1% (YNR030W) identity with YGL142C. The hydrophobicity plot of YNR030W is particularly similar to the one of YGL142C. All of these genes

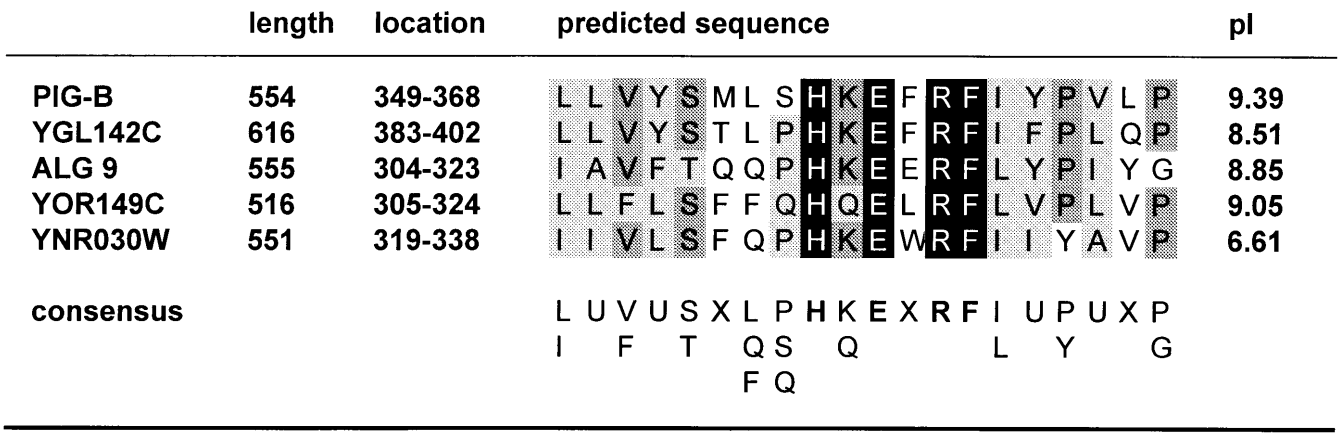

Fig. 9. PIG-B, YGL142C, and related yeast sequences share a common sequence motif. PIG-B and three yeast homologs were found through a BLAST search and aligned using the pileup multiple alignment program of the GCG software package (http://gcg.ch.embnet.org:8080/htdocs). Conservative replacements are listed below the consensus sequence; 100% identity is marked in black, 80% in gray; 60% identity and conservative substitutions are in light gray. The length of the open reading frame is given on the left. X designates any amino acid; U indicates amino acids with unpolar side chains; pI, isoelectric point. Sequences and pIs are taken from the YPD (http://quest7.proteome.com/YPDhome.html) or NCBI databases (http://www.ncbi.nlm.nih.gov/) except PIG-B (Takahashi *et al.*, 1996).

contain a highly conserved motif which is intercalated between two hydrophobic regions (Figure 9). A Blast search (Altschul *et al.*, 1990) shows that this "HXEXRF" motif does not seem to be present in any other yeast open reading frame. The extreme hydrophobicity of YGL142C and PIG-B may be a general feature of glycosyltransferases which use dolichol-linked hexoses as a substrate. Indeed, *ALG6* and *ALG8*, two α1,3-glucosyltransferases adding Glc from Dol-P-Glc onto the lumenally oriented Dol-PP-GlcNAc$_2$-Man$_9$ intermediate as well as *ALG3* which adds Man from Dol-P-Man onto Dol-PP-GlcNAc$_2$-Man$_5$ (Aebi *et al.*, 1996) all are very hydrophobic proteins. All known proteins involved in the transfer of dolichol-linked hexoses also have a relatively high isoelectric point between 8.5 and 9.6 (Figure 9).

Previous experiments have shown that the N-terminal, cytosolic extension of PIG-B is not essential for its function (Takahashi *et al.*, 1996) but it seems difficult at present to predict at what side of the membrane the active center of this protein is localized. The homology with *ALG9* may indicate that it is on the lumenal side but the very hydrophobic character of this class of transferases may indicate that they are additionally involved in the transmembrane movement of the Dol-P-Man or Dol-P-Glc substrates.

Further experiments hopefully will allow to better understand the exact function and orientation of these proteins.

Materials and methods

Strains, growth conditions, and materials

Wild type *S.cerevisiae* strains were W303–1A (*Mata ade2–1 can1–100 ura3–1 leu2–3,112 trp1–1 his3–11,15*), W303–1B (*Matα ade2–1 can1–100 ura3–1 leu2–3,112 trp1–1 his3–11,15*) and X2180–1A (*Mata lys*$^-$). GPI-mutants (Benghezal *et al.*, 1995) were FBY11 (*Matα ade2–1 can1–100 ura3–1 leu2–3,112 trp1–1 his3–11,15 gpi8–1*). The original mutant 839 which we named FBY169 (*Matα ade2–1 can1–100 ura3–1 leu2–3,112 trp1–1 his3–11,15 gpi10–1*) and the out and back crossed strain FBY618 (*Matα trp1–1 gpi10–1*). For labeling with [2–^{3}H]Man we used C4 (*Mata leu2–3,112 ura3–52 pmi40*) (Payton *et al.*, 1991). Maintenance and growth conditions have recently been described previously (Benghezal *et al.*, 1996).

Reagents were obtained from the following sources: *myo*-[2–^{3}H]inositol (15 Ci/mmol), uridine diphosphate-*N*-acetyl-D-glucosamine [glucosamine-6–^{3}H] (40 Ci/mmol), [1–^{3}H]ethanolamine (30 Ci/mmol), and [2–^{3}H]Man (20 Ci/mmol) were from Anawa Trading SA (Zürich, Switzerland); EN^{3}HANCE from NEN (Hertfordshire, UK); 0.2-mm-thick silica gel 60 plates (20 × 20 cm) and phenylmethylsulfonyl fluoride (PMSF) from Merck (Darmstadt, Germany); nikkomycin Z from Calbiochem (San Diego, CA); ATP, coenzyme A, GDP-mannose, lyticase, and tunicamycin from Sigma (St. Louis, MO); leupeptin, pepstatin, chymostatin, and antipain from Fluka (Buchs, Switzerland); 5-fluoroorotic acid (5-FOA) from Toronto Research Chemicals Inc. (Ontario, Canada); ammonia in methanol (7 M) from Janssen Chimica (Beerse, Belgium); zymolase 20T from Seikagaku Corp. (Tokyo, Japan); jack bean-α-mannosidase from the Oxford Glycosystems (Oxford, UK); mixed bed ion exchange resin (AG-501-X8) from Bio-Rad Laboratories (Hercules, CA); phosphatidylinositol-specific phospholipase C (PI-PLC) from Boehringer Mannheim (Mannheim, Germany); 300-fold purified GPI-phospholipase D (GPI-PLD) from bovine serum was a kind gift of Dr. U. Brodbeck, University of Bern (Bern, Switzerland).

Plasmids

The pBF61 vector was constructed by PCR amplification of the full length YGL142C sequence from genomic DNA with the following primers: IIP-1F (5′-GCTGTCTGTTGCCTAAAGA-3′) starting 579 bp upstream of the start codon and IIP-2R (5′-CACCCTTAAAGATGGACCT-3′) starting 359 bp downstream of the stop codon, using the Expand High Fidelity PCR System from Boehringer Mannheim. The PCR fragment was digested with *Pst*I and *Stu*I and subcloned into the *Pst*I/*Sma*I site of the YEplac195 multicopy vector (Gietz and Sugino, 1988). pBF62 was prepared by subcloning the *Pst*I/*Kpn*I YGL142C fragment of pBF61 into the multiple cloning site of the

centromeric vector YCplac33 (Gietz and Sugino, 1988). To construct pBF64 the same YGL142C fragment was ligated into YIplac211 (Gietz and Sugino, 1988) digested with *Pst*I/*Kpn*I. YGL142C including 514 and 111 bp of the 5′ and 3′ untranslated regions, respectively, was amplified by PCR from genomic DNA of *gpi10–1* as it has been done for the wild type YGL142C by using the Expand High Fidelity PCR System. Both strands of two independent PCR reactions were sequenced for us by Microsynth GmbH (Balgach, Switzerland).

Protoplast fusion

Fusion of the yeast strain FBY169 with X2180–1A was done as described previously (Tsuboi, 1981) with the following modifications: cells were cultured at 24°C and converted to protoplasts with lyticase under gentle agitation during 30–60 min at 24°C. For fusion 5×10^8 protoplasts of each fusion partner were mixed. Fused protoplasts were resuspended in 6 ml regeneration agar (45°C) (Hinnen *et al.*, 1978) and poured onto selective plates.

Metabolic radiolabeling and lipid extraction

Cells were grown in SDCUA medium. Exponentially growing cells were centrifuged, washed and resuspended in fresh SDCUA medium for labeling at a density of 10 OD_{600} units/ml. Cells (2.5 OD_{600} units in 250 µl of SDCUA) were preincubated for 20 min and labeled with [^{3}H]Ins (5 µCi) for 120 min whereby the cells were diluted 4-fold with SDCUA 40 min after the addition of [^{3}H]Ins. Labeling of cells with [^{3}H]EtN was done as described previously (Menon and Stevens, 1992) with a few minor modifications: 10 OD_{600} of cells were resuspended in 1 ml of SDCUA, preincubated for 10 min at 37°C, 50 µCi of radiolabel were added and cells incubated in a shaking water bath. After 2 h cells were diluted with one volume of fresh medium and incubated for 2 more hours. Strain C4 was labeled with [2–^{3}H]Man as described previously (Sipos *et al.*, 1994). To stop the labeling cells were placed on ice and NaN_3 and NaF (10 mM final) were added. Cells were washed with 1 M sorbitol at 4°C. Lipids were extracted and desalted by butanol extraction as described previously (Sipos *et al.*, 1994). Lipid extracts were analyzed by ascending TLC using 0.2 mm thick silica gel 60 plates with the solvent 1 (chloroform/methanol/water; 10/10/3, v/v/v) or solvent 2 (chloroform/methanol/0.25% KCl; 55/45/10, v/v/v). Radioactivity was detected by two-dimensional radioscanning (LB 2842; Berthold AG, Regensdorf, Switzerland) or else, TLC plates were sprayed with EN^{3}HANCE and exposed for fluorography at -80°C.

Preparation of yeast membranes

Membranes were prepared as described previously (Goud *et al.*, 1988) from exponentially growing cell cultures in SDCUA medium. Cells were converted to spheroplasts and homogenized as described previously with the exception that the cells were incubated for 1 h in 0.2 mg/ml of zymolase and spheroplasts were broken by forcing them through a 0.4×19 mm syringe at 4°C in lysis buffer. Intact cells were eliminated by low speed centrifugation and membranes were obtained by two centrifugations at $15{,}000 \times g$ for 30 min at 4°C and resuspended at about 10 mg protein/ml in lysis buffer (Goud *et al.*, 1988).

Purification of GPI-peptides

GPI proteins were extracted and extensively delipidated with organic solvents and were further delipidated by Concanavalin A–Sepharose affinity chromatography; GPI peptides were obtained by pronase digestion and purified over octyl-Sepharose exactly as described previously (Sipos *et al.*, 1997).

Biosynthesis of GPIs by microsomes in vitro

In vitro biosynthesis of GPIs using UDP-[^{3}H]GlcNAc was performed as described (Costello and Orlean, 1992). All reactions were carried out at 37°C and in a final volume of 100 µl. Aliquots of freshly prepared membranes (100 µg protein/tube) were diluted into 100 mM TRIS-HCl, pH 7.5, 1 mM EGTA, 3 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM ATP, 1 mM Coenzyme A, 21 µg/ml tunicamycin, and 10 µM nikkomycin Z (to inhibit utilization of UDP-GlcNAc by chitin synthases (Dähn *et al.*, 1976) and in some cases 1 mM GDP-Man. Membranes were preincubated for 10 min at 37°C and then transferred to a tube containing 6 µCi dried UDP-[^{3}H]-GlcNAc. After incubation for 1 h the reaction was stopped by the addition of 1 ml chloroform/methanol (1/2, v/v). After thorough mixing and removal of insoluble components by centrifugation, the supernatant was saved and the pellet extracted a second time with 0.5 ml of solvent 1. The lipids from both extractions were pooled, dried and partitioned between butanol and water to remove salts. Lipid extracts were analyzed as mentioned above.

Structural analysis of GPIs

PI-PLC, GPI-PLD, and JBAM (jack bean α-mannosidase) treatments were carried out at 37°C following procedures described previously (Puoti and Conzelmann, 1993; Davitz *et al.*, 1989; and Ralton *et al.*, 1993, respectively).

Soluble head groups were obtained from purified glycolipids or anchor-peptides through selective removal of acyl groups attached to the Ins using methanolic NH_3 (Roberts *et al.*, 1988a) followed by PI-PLC treatment in 20% propanol (Puoti and Conzelmann, 1993). Fatty acids and lipids were removed by butanol extraction. Head groups were subjected to hydrofluoric acid (HF) dephosphorylation (Ferguson *et al.*, 1988) and were N-acetylated (Gopal and Ballou, 1987) and desalted using mixed-bed ion exchange resin AG-501-X8 (Puoti and Conzelmann, 1992). Acetolysis and JBAM treatments were done as described previously (Schneider *et al.*, 1990).

N-Acetylated, water-soluble head group fragments were separated by ascending TLC using 0.2-mm-thick silica gel 60 plates in four consecutive runs in butanol/ethanol/water (4/3/3, v/v/v; Puoti and Conzelmann, 1993) and analyzed by fluorography as described above or were separated by ascending paper chromatography in methylketone/pyridine/water (20/12/11, v/v/v) as described previously (Sipos *et al.*, 1994).

Analysis of dolichol-linked oligosaccharides

Cells were labeled with [^{3}H]Man, lipids were extracted, oligosaccharides released and analyzed by HPLC exactly as described previously (Zufferey *et al.*, 1995).

Note added in proof

The mutant gene *gpi10-1* (encoding Thr at position 48) and the same gene corrected according to the wild type gene (encoding Ala at position 48) were cloned into YCplac33 vector to generate

pBF67 and pBF69, respectively. After transformation of *gpi10-1* with these two plasmids, cells were labeled with ^{3}H-Ins and the lipids were analyzed. pBF67 does not abolish the M2* accumulation of *gpi10-1* whereas pBF69 does. This result demonstrates that the mutation (Thr48) is responsible for the phenotype of M2* accumulation in *gpi10-1* cells.

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Abbreviations

CP, complete precursor; EtN, ethanolamine; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; HF, hydrofluoric acid; Ins, *myo*-inositol; IPC, inositolphosphoceramide; JBAM, jack bean α-mannosidase; MIPC, mannosylinositolphosphoceramide; $M(IP)_2C$, inositolphospho-MIPC; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; TLC, thin layer chromatography.

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