Ethanolaminephosphate Side Chain Added to Glycosylphosphatidylinositol (GPI) Anchor by Mcd4p Is Required for Ceramide Remodeling and Forward Transport of GPI Proteins from Endoplasmic Reticulum to Golgi*

Yonghua Zhu1, Christine Vionnet, and Andreas Conzelmann2

From the Department of Medicine/Biochemistry, University of Fribourg, CH-1700 Fribourg, Switzerland

Glycosylphosphatidylinositol (GPI) anchors of mammals as well as yeast contain ethanolaminephosphate side chains on the α1–4- and the α1–6-linked mannoses of the anchor core structure (protein-CO-NH-(CH2)2-PO4-6Man1–2Manα1–6Manα1–4GlcNH2-inositol-PO4-lipid). In yeast, the ethanolaminephosphate on the α1–4-linked mannose is added during the biosynthesis of the GPI lipid by Mcd4p. MCD4 is essential because Gpi10p, the mannosyltransferase adding the subsequent α1–2-linked mannose, requires substrates with an ethanolaminephosphate on the α1–4-linked mannose. The Gpi10p ortholog of Trypanosoma brucei has no such requirement. Here we show that the overexpression of this ortholog rescues mcd4Δ cells. Phenotypic analysis of the rescued mcd4Δ cells leads to the conclusion that the ethanolaminephosphate on the α1–4 linked mannose, beyond being an essential determinant for Gpi10p, is necessary for an efficient recognition of GPI lipids and GPI proteins by the GPI transamidase for the efficient transport of GPI-anchored proteins from the endoplasmic reticulum to Golgi and for the physiological incorporation of ceramides into GPI anchors by lipid remodeling. Furthermore, mcd4Δ cells have a marked defect in axial bud site selection, whereas this process is normal in gpi7Δ and gpi1Δ. This also suggests that axial bud site selection specifically depends on the presence of the ethanolaminephosphate on the α1–4-linked mannose.

The carbohydrate structure linking the C-terminal end of glycosylphosphatidylinositol (GPI)3 anchored proteins to the lipid moiety is identical in GPI anchors from all organisms analyzed so far, but the GPI anchors from various species differ widely with regard to the side chains attached to this core structure as well as the lipid moieties of the anchor (1, 2). This report concerns the ethanolaminephosphate (EtN-P) side chain, which is often present on mannose 1 of the core structure (Man1 in Fig. 1). Indeed, an EtN-P is found on Man1 of GPI lipids and GPI proteins in mammals, Saccharomyces cerevisiae, and Torpedo californica, and possibly Candida albicans, but is not found in other organisms such as Trypanosoma brucei, Leishmania major, or Plasmodium falciparum (3–7). The EtN-P transferred transferring EtN-P to Man1 is encoded by MCD4 in yeast and its ortholog PIG-N in mammals (Fig. 1) (8, 9). Phosphatidylethanolamine serves as a donor of the EtN-P group (10). Yeast also possesses two MCD4 homologs, GPI7 and GPI13, which are involved in the transfer of EtN-P to Man2 and Man3, respectively (Fig. 1). MCD4 is essential and can be inhibited by YW3548 (6). Addition of this inhibitor or the depletion of Mcd4p arrest the growth of yeast cells and lead to the accumulation of the abnormal GPI lipid 4c (Man1–6Manα1–4GlcNH2-inositol-PO4-lipid), indicating a problem with the addition of Man3 (Fig. 1) (6, 7, 9, 11). This suggested that Gpi10p strongly prefers substrates carrying an EtN-P substituent on Man1. Indeed, overexpression of Mcd4p can improve the growth of yeast cells in the presence of low concentrations of YW3548, whereas overexpression of Gpi10p is comparatively inefficient (9, 11). Although these data indicate that Mcd4p helps Gpi10p by providing an optimal substrate, they cannot rule out the possibility that MCD4 would influence the function of Gpi10p in another way, e.g. by regulating phosphatidylethanolamine levels in the ER or by channeling substrate into Gpi10p or stabilizing Gpi10p.

There indeed is a genetic link between MCD4 and phosphatidylethanolamine biosynthesis: the mcd4–P301I and mcd4–174 alleles render cells temperature-sensitive (ts) when combined with psd1Δ, with the latter eliminating the phosphatidylethanolamine decarboxylase, by which the bulk of phosphatidylethanolamine is made. The mcd4–P301I mutation was claimed to not affect GPI biosynthesis. Temperature sensitivity of mcd4 psd1Δ mutants could be reverted by the addition of ethanolamine, or choline, or ethanolamine and sorbitol to the media (12). Temperature sensitivity of another mcd4 allele named fsr2–1 is suppressed by overexpression of PSD1, PSD2, or ECM33 (13).

Mutations in MCD4 also show several other, quite specific phenotypes, which may not be related to its effect on GPI biosynthesis. Mutants in MCD4 (mitotic check point dependent) were found to suffer from delayed bud emergence and an inabil-

* This work was supported by Swiss National Science Foundation Grant 31-67188.01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Institute of Live Science and Biotechnology, Hunan University, 410082 Changsha City, China.

2 To whom correspondence should be addressed: Div. of Biochemistry, Chemin du Musée 5, CH-1700 Fribourg, Switzerland. Tel.: 41-26-300-8630; Fax: 41-26-300-9735; E-mail: andreas.conzelmann@unifr.ch.

3 The abbreviations used are: GPI, glycosylphosphatidylinositol; CHX, cycloheximide; EtN-P, ethanolaminephosphate; KO, knock-out; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; ts, temperature-sensitive; wt, wild type; ER, endoplasmic reticulum.
ity to polarize secretion to the bud tip or septum, thus becoming dependent on a checkpoint delaying mitosis until a bud is formed (14). Buds of mcd4–174 are small and misshapen and contain many aberrant membrane structures (8). Certain mcd4 mutants also activate one branch of the hyperosmotic response pathway and are more resistant to high concentrations of Cu^{2+} than wt cells (15).

GPI lipids and GPI anchors of T. brucei do not contain any EtN-P side chains, and thus, their presence certainly will not be required by the T. brucei GPl10 ortholog (3, 16). To test the relationship between MCD4 and GPl10 and explore the various functions of MCD4 more closely, we tested whether it was possible to overcome the lethality of the mcd4Δ deletion by introducing T. brucei GPl10p into mcd4Δ cells. The results show that in this way it is possible to create a cell in which all functions of MCD4 are lost while some GPI biosynthesis is preserved.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Conditions, and Materials**—The S. cerevisiae strains used were: MCD4/mcd4Δ, MATα/α his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 MET17/met17Δ0 ura3Δ0/ura3Δ0 YKL165c::kanMX4/YKL165c (EUROSCARF); T1, MATα his3Δ1 leu2Δ0 ura3Δ0 p425-TbGPl10; T2, MATα his3Δ1 leu2Δ0 ura3Δ0 met17Δ0 p425-TbGPl10; T3, MATα his3Δ1 leu2Δ0 ura3Δ0 mcd4::kanMX4 p425-TbGPl10; T4, MATα his3Δ1 leu2Δ0 ura3Δ0 met17Δ0 mcd4::kanMX4 p425-TbGPl10; MCD4 (17A-H42), MATα trp1–289 ura3–52 leu2; mcd4Δ (521–17A-H42), MATα trp1–289 ura3–52 leu2 ssu21 (17); MKY3, MATα trp1 lys2 ura3 leu2 his3 psd1Δ1::TRPl mcd4Δ-p301L etn−1 (ethanolamine-dependent at 37 °C) and MKY13, MATα arg4 ura3 his3 trp1 leu2 psd1Δ1::TRPl mcd4Δ–174 (12); BY4742, MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (EUROSCARF); yap3Δ, YLR120c::kanMX4 in BY4742 (EUROSCARF); gpi8 (164–161), MATα his4 leu2; gpi8 sec18 (FYB91), gpi8–1 sec18–1Δ leu2–3,112 (7); gpi1Δ, MATα ura3–52 his3::hisG gpi1::URA3 pRS452-CCW212HA; gpi1, MATα ura3–52 lys2 gpi1 (isolated as cwh4 by Frans Klis); gpi7Δ, YJL062w::kanMX4 in BY4742 (EUROSCARF); the cells were grown on rich medium (YPD) or minimal medium (SD) (18) containing 2% glucose (D) as a carbon source and uracil (U), adenine (A), and amino acids (aa) as required at 30 °C. Methionine was added at 10 mg/liter (38 μM), so that the MET17 promoter was almost fully active. Oligonucleotide synthesis and DNA sequencing services were provided by Microsynth (Balgach, Switzerland). Verification of deletions was done by PCR as shown in Fig. 2C using the following primers: F1, 5’-ATGTGGAAACAAAACCAAGAGCA-3’; F2, 5’-CACACCGTGGATTCTGTTACAC-3’; R1, 5’-GCTGATGAAGATTCGGC-3’; R2, 5’-AGCATAATCAGACATGACC-3’; R3, 5’-CAAGGAGGTTATCTTGGGC-3’; F4, 5’-CCGGATCCTAGCCTGTTGTTGATTTCCC-3’; and R4, 5’-CGGAATTCGCTACTTGTCATCCGATCC.

**Construction of Yeast Vectors**—The T. brucei GPl10 behind the MET17 promoter on a multicopy plasmid (p425-TbGPl10, LEU2) was a kind gift from Howard Riezman and Taroh Kinoshita (16). The promoter and open reading frame of MCD4 was amplified by PCR and inserted into YEp352 to yield pBF639 (URA3). Sequence verification showed a point mutation I387T (at DNA level, T1160C), but the plasmid was able to fully complement mcd4Δ mutants.

**Cell Extraction and Western Blotting**—The proteins were extracted by incubating cells for 5 min in NaOH and boiling at 95 °C in reducing sample buffer (19). Alternatively, the cells were broken by glass beads in TEPI buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 30 μg/ml of each, leupeptin, antipain, and pepstatin, 2 mM phenylmethylsulfonyl fluoride) at 4 °C, and the lysate was directly solubilized in octyl-β-glucoside (50 mM) at 4 °C for 30 min. After solubilization, the extracts were centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant was precipitated with trichloroacetic acid and boiled in reducing sample buffer as above. In other experiments, the cells were broken by glass beads in TEPI buffer, the lysate was directly treated with DNase (Fluka 31136) for 45 min at room temperature, and then the lysate was solubilized with Triton X-114 (1%) at 4 °C for 30 min. After solubilization, insoluble material was removed by centrifugation at 16,000 × g for 5 min at 4 °C. The supernatants were separated into detergent (D) and aqueous (A) phases at 37 °C followed by mild centrifugation. The detergent and aqueous fractions were then precipitated with trichloroacetic acid. For treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), the detergent phase was diluted and treated with PI-PLC from Bacillus cereus (Sigma P 5542 or Funakoshi Pharm BR-05) using 1 unit/ml at 37 °C for 15 min with occasional shaking. After a further phase separation the detergent and aqueous phases were precipitated with trichloroacetic acid and processed as above. As primary antibodies we used purified mouse anti-CPY (Molecular Probes), rabbit anti-Yap3p (a kind gift from Dr. Niamh Cawley), and rabbit anti-Gas1p made in the laboratory. As secondary antibodies for blots, we used fluorescent goat anti-mouse IgG conjugated to AlexaFluor680 or goat anti-rabbit IgG conjugated to IRDye800, for treatment with specific phosphatidylinositol-specific phospholipase C (PI-PLC), the detergent phase was diluted and treated with PI-PLC from Bacillus cereus (Sigma P 5542 or Funakoshi Pharm BR-05) using 1 unit/ml at 37 °C for 15 min with occasional shaking. After a further phase separation the detergent and aqueous phases were precipitated with trichloroacetic acid and processed as above. As primary antibodies we used purified mouse anti-CPY (Molecular Probes), and rabbit anti-Gas1p made in the laboratory. As secondary antibodies for blots, we used fluorescent goat anti-mouse IgG conjugated to AlexaFluor680 or goat anti-rabbit IgG conjugated to IRDye800, and fluorescence was measured with the Odyssey Imaging System (LI-COR). Alternatively, the blots were revealed with goat anti-rabbit IgG conjugated to peroxidase and using ECL technology (Amersham Biosciences).

**Analysis of GPls**—GPl lipids were analyzed by metabolic labeling of yeast cells with [3H]myo-inositol and labeling of yeast microsomes with UDP-[3H]GlcNAc as described before (7). Head groups of GPI lipids were analyzed with hydrofluoric acid using the protocol originally developed by Mike Ferguson (3), and remodeling of lipids on GPI anchors was analyzed by
**Ethanolaminephosphate on Yeast GPI Anchors**

A T1 T2 T3 T4

C F2 F1 MCD4

F2 R2 R1 mcd4Δ::KanMX

F4 p425MET17-TbGPI10

F3

P T1 T2 T3 T4

F1/R1

F2/R3

F2/R2

F4/R4

D T2 T4

+ EtN/Ins/C + Sor + Sor/En/Ins/C

FIGURE 2. *T. brucei* Gpi10p Can rescue viability and growth of yeast mcd4Δ cells. A, the (mcd4Δ/MCD4) diploid strain was transformed with p425-TbGPI10 carrying TbGPI10 under the MET17 promoter and was sporulated. One complete tetrad is shown. B, the genotype of strains T1–T4 derived from the tetrad shown in A was evaluated by plating on methionine drop out and G418 plates. C, PCR analysis of the DNA of strains T1–T4 and the parental mcd4Δ/MCD4 diploid (P). The picture shows the homology regions of primers on the target DNA and the results of the PCR reactions done with various primer combinations as indicated. D, 10-fold dilutions of T2 or T4 cells were plated on SDaaUA plates containing 2 mM EtN, 50 mg/liter inositol (Ins), 2 mM choline (C), 1 M sorbitol (Sor) alone or in combination. The plates were incubated at 37 °C for 6 days.

liberating anchor lipids from anchor peptides using HNO₂ as described (20).

**Microscopy**—To stain cells with calcofluor white, cells were kept in continuous exponential growth for >10 generations, and one A600 unit of cells was washed with water, stained in calcofluor white (1 mg/ml) for 10–20 min at room temperature, washed again, and observed under the microscope.

**RESULTS**

*T. brucei* Gpi10p Can Rescue Viability of mcd4Δ Cells—The multicopy vector p425-TbGPI10 harboring TbGPI10 behind the MET17 promoter was introduced into a mcd4Δ::KanMX/MCD4 heterozygous diploid strain. The transformed diploid was sporulated, and tetrads were dissected. Very few complete tetrads were obtained. Fig. 2A shows a complete tetrad, in which geneticin resistance and methionine auxotrophy segregated 2:2 (Fig. 2B), suggesting that spores T3 and T4 were mcd4Δ::KanMX. PCR analysis confirmed the mcd4Δ::KanMX genotype and the presence of the p425-Tb-GPI10 plasmid in T3 and T4 (Fig. 2C). The mcd4Δ::KanMX strains grew rather slowly. The generation times at 30 °C in liquid SDaaUA medium of T1, T2, T3, and T4, were 2.48, 2.39, 5.25, and 6.03 h, respectively. The corresponding generation times in YPDUA were 1.53, 1.61, 7.38, and 6.15 h, respectively (not shown). The MET17 promoter driving TbGPI10 expression is three to five times more active in the absence of methionine (21). Omission of methionine from minimal medium to achieve a higher expression of TbGPI10 did not enhance the growth rate of MET17 mcd4Δ::KanMX cells (not shown). On plates the mcd4Δ/TbGPI10 cells grew at almost the same rate at 24 °C, 30 °C and 37 °C, but they were inositol-dependent at 37 °C (Fig. 2D). The growth rate of mcd4Δ/TbGPI10 cells on plates at 37 °C was also increased by 1 M sorbitol and slightly improved by ethanolamine, and the greatest effect on colony size was observed when all these ingredients were combined (Fig. 2D). Sorbitol has been reported to rescue certain mcd4 mutants (12, 22). However, the p425-TbGPI10 plasmid did not allow various temperature-sensitive mcd4Δ strains (ssu21, mcd4Δ–174, and mcd4Δ-P301L) to grow at restrictive temperature (37 °C) (not shown), neither in the presence nor the absence of ethanolamine. This may suggest a possible dominant negative effect of these mutant alleles.

The mcd4Δ/TbGPI10 Cells Accumulate an Abnormal GPI Lipid—To investigate the structure of the GPI lipids made by mcd4Δ/TbGPI10 strains, we labeled intact cells with [3H]inositol and analyzed the lipid extracts by TLC and fluorography. In wild type (wt) cells, no GPI lipids can be detected in this way, but certain mutants deficient in GPI biosynthesis or GPI addition to proteins were shown to accumulate significant amounts of labeled biosynthetic intermediates (23). As seen in Fig. 3A, there is an abnormal lipid accumulating in T3 and T4 mcd4Δ/TbGPI10 strains, which is absent from the T1 and T2 MCD4 strains (lanes 5 and 6 versus lanes 2 and 3). This lipid was named M4⁺ because it has about the same mobility as M4/2, which is one of the several abnormal GPI lipids accumulating in GPI transamidase mutants such as gpi8 sec18 (Fig. 3A, lane 7). Although CP2 contains an EtN-P group on Man1, Man2, and Man3 (Fig. 1), gpi7Δ mutants accumulate M4/1 (Fig. 3A, lane 4), which lacks the EtN-P on Man2 while preserving the EtN-Ps on Man1 and Man3 (23). The head group of M4⁺ could be released by...
Ethanolaminephosphate on Yeast GPI Anchors

when these cells expressed TbGPI10 and independent of the presence or absence of ethanolamine in the labeling medium (not shown). This again points to a possible dominant negative effect of these mcd4 alleles.

To further investigate the GPI biosynthesis pathway, we prepared microsomes and incubated them with UDP-[3H]GlcNAc. As shown in Fig. 3C, the mature GPI lipid CP2 (Fig. 1) was not detectable in mcd4Δ/TbGPI10 strains T3 and T4 (lanes 3 and 4). However, mcd4Δ/TbGPI10 strains showed an abnormal accumulation of lipid 4c (Fig. 3C, lanes 3 and 4, open triangles), which also accumulates in a temperature-sensitive mcd4 mutant at 37 °C (Fig. 3C, lane 8) and which has previously been characterized as Man-Man-GlcN-(acyl→)PI (7). The mcd4Δ/TbGPI10 strains do not accumulate lipid 031b (Man-Man-Man-(EtN-P→)Man-GlcN-(acyl→)PI), an intermediate containing four mannoses and accumulating in Gpi13p-depleted strains (24), and they make no M4/1 (Man-(EtN-P→)Man-Man-(EtN-P→)Man-GlcN-(acyl→)PI), typical of gpi7Δ strains (Fig. 3C, lane 7). The presence of the abnormal lipid 4c in mcd4Δ/TbGPI10 indicated that TbGPI10 was rather inactive under the in vitro conditions used here. This is confirmed by the fact that the gpi10Δ/TbGPI10 strain accumulates M2* (Fig. 3C, lane 6, open circle), a lipid previously characterized as Man-(EtN-P→)Man-Man-GlcN-(acyl→)PI (25).

Interestingly, traces of a lipid migrating to the position of M4* are visible in T1 and T3, but not T2 and T4, and the same lipid is also present in gpi10Δ/TbGPI10 (Fig. 3C, lanes 1, 3, and 6, asterisk). This potential M4* correlate was only observed in microsomes from MET17, not in met17ΔA strains, and the difference may be related to different expression levels of TbGpi10p.)

Incorporation of [3H]inositol into proteins is a measure of GPI protein biosynthesis and was found to be markedly decreased in mcd4Δ/TbGPI10 (Fig. 3D). Most affected were the mature proteins, which, after glycan elongation in the Golgi, appeared as a high molecular weight smear. Their absence suggests that mcd4Δ/TbGPI10 cells may have difficulty in transporting proteins to the Golgi (see below).

mild deacylation followed by PI-PLC treatment, and, as shown in Fig. 3B, treatment of the liberated head group with hydrofluoric acid and acetic anhydride yielded a Man₄-GlcNAc-Inositol fragment. This partial analysis of lipid M4* is compatible with the idea that M4* is a CP2 lacking EtN-P on Man1, thus being the most mature GPI lipid we theoretically can expect in the GPI protein biosynthesis pathway, we prepared microsomes and incubated them with UDP-[3H]GlcNAc. As shown in Fig. 3C, the mature GPI lipid CP2 (Fig. 1) was not detectable in mcd4Δ/TbGPI10 strains T3 and T4 (lanes 3 and 4). However, mcd4Δ/TbGPI10 strains showed an abnormal accumulation of lipid 4c (Fig. 3C, lanes 3 and 4, open triangles), which also accumulates in a temperature-sensitive mcd4 mutant at 37 °C (Fig. 3C, lane 8) and which has previously been characterized as Man-Man-GlcN-(acyl→)PI (7). The mcd4Δ/TbGPI10 strains do not accumulate lipid 031b (Man-Man-Man-(EtN-P→)Man-GlcN-(acyl→)PI), an intermediate containing four mannoses and accumulating in Gpi13p-depleted strains (24), and they make no M4/1 (Man-(EtN-P→)Man-Man-(EtN-P→)Man-GlcN-(acyl→)PI), typical of gpi7Δ strains (Fig. 3C, lane 7). The presence of the abnormal lipid 4c in mcd4Δ/TbGPI10 indicated that TbGPI10 was rather inactive under the in vitro conditions used here. This is confirmed by the fact that the gpi10Δ/TbGPI10 strain accumulates M2* (Fig. 3C, lane 6, open circle), a lipid previously characterized as Man-(EtN-P→)Man-Man-GlcN-(acyl→)PI (25).

Interestingly, traces of a lipid migrating to the position of M4* are visible in T1 and T3, but not T2 and T4, and the same lipid is also present in gpi10Δ/TbGPI10 (Fig. 3C, lanes 1, 3, and 6, asterisk). This potential M4* correlate was only observed in microsomes from MET17, not in met17ΔA strains, and the difference may be related to different expression levels of TbGpi10p.)

Incorporation of [3H]inositol into proteins is a measure of GPI protein biosynthesis and was found to be markedly decreased in mcd4Δ/TbGPI10 (Fig. 3D). Most affected were the mature proteins, which, after glycan elongation in the Golgi, appeared as a high molecular weight smear. Their absence suggests that mcd4Δ/TbGPI10 cells may have difficulty in transporting proteins to the Golgi (see below).
proteins. The mcd4Δ/TbGPI10 cells release Gas1p into the medium (Fig. 4B), and this phenomenon is only partially explained by cell lysis, because relatively less immature Gas1p is found in the medium than in the cells. Release of mature GPI proteins could be shown more clearly for Yap3p (see below).

To assay the rate of transport of immature Gas1p to the Golgi, the amount of immature Gas1p was also determined after incubation of cells with cycloheximide (CHX). As shown in Fig. 5A, the relative amounts of immature Gas1p (sum of aqueous and detergent phases) is reduced 5-fold during a 20-min incubation in the presence of CHX in T1 wt cells (from 10% down to 2%), whereas in the mutant T3 only a small fraction of immature Gas1p disappears (from 49% down to 40%). Thus, immature Gas1p seems to be transported only very slowly out of the ER in the mcd4Δ/TbGPI10 mutant. As mentioned above, GPI proteins accumulate in the ER when they fail to be anchored but accumulation of GPI proteins in the ER is also observed in other instances; the transport of GPI proteins to the Golgi also fails if COPI, or sphingolipid biosynthesis, or the Emp24p complex, or yet other factors are deficient (for review see Ref. 29). To see whether the immature Gas1p accumulating in mcd4Δ/TbGPI10 is anchored or not, we partitioned Gas1p before and after PI-PLC treatment in the detergent Triton X-114 (30). Fig. 5B indicates that the percentage of immature Gas1p that partitions into the aqueous phase because of the action of PI-PLC treatment is higher in mcd4Δ/TbGPI10 than in wt cells (62 versus 50%). (It should be noted that it is difficult to shift the total of GPI proteins into the aqueous phase by this technique. In Fig. 5B, even for the mature, GPI-anchored form, only a fraction could be shifted, namely 37% in wt and 42% in mcd4Δ/TbGPI10). Importantly, because the percentages of Gas1p shifted by PI-PLC from detergent to aqueous phase are higher for immature than for mature Gas1p, there is no indication that immature, nonanchored Gas1p would be accumulating, neither in wt nor mcd4Δ/TbGPI10 mutant cells. There are reports showing that during Triton X-114 solubilization at 4 °C, part of GPI-anchored proteins are recovered in the detergent-insoluble pellet. The efficiency of solubilization in our experiment was evaluated by probing equivalent samples of the detergent-insoluble pellet and the detergent extract (Fig. 5C). Although Triton X-114 only solubilized 62 and 80% of Gas1p in wt and mutant cells, respectively, immature Gas1p was solubilized nearly quantitatively in both strains. Thus, the results obtained in Fig. 5 (A and B) are representative for the total of immature Gas1p. Pulse-chase analysis confirmed the severe transport block of Gas1p (Fig. 5D).

To generalize the data obtained with Gas1p, we also investigated the fate of another GPI protein, Yap3p (Yps1p). Yap3p, a 570-amino acid-long protein containing 10 potential N-glycosylation sites was described as a 68-kDa GPI-anchored protein (31), but in other reports using the same antibodies, the immature forms of this protein ran as 80-kDa (32) or as 80- and 100-kDa proteins (33) or as bands at 85 and 115 kDa (34), whereas mature forms ran as a broad smear around 150–200 kDa. As can be seen in Fig. 6A, the mcd4Δ/TbGPI10 mutant (T4) contained much less of the mature (140–240 kDa) Yap3p form than the wt (T2) strain or the

---

**FIGURE 4.** The mcd4Δ/TbGPI10 cells contain reduced amounts of mature Gas1p. A, T1–T4 cells and the parental BY4742 cells were cultured in YPDUA at 30 °C, protein extracts were prepared (19) and processed for Western blotting using antibodies against Gas1p and CPY and developed using the LI-COR system. The positions of mature (m), immature (i), and unprocessed precursors (p1 and p2) are indicated. B, cells were grown in YPDUA at 30 °C, media (M) and cell lysates (C) (solubilized with octyl-β-glucoside) were processed for Western blotting using anti-Gas1p antibodies and the ECL system.

Immature GPI Proteins Accumulate in the mcd4Δ/TbGPI10—
To see whether GPI lipids are transferred onto GPI proteins at a normal rate, we analyzed the GPI protein Gas1p, whose ER form migrates at 105 kDa and whose mature form, produced by elongation of N- and O-glycans upon arrival in the Golgi, migrates at 125 kDa in SDS-PAGE. As long as a GPI anchor is not added to Gas1p in the ER, the protein is not packaged into COPII vesicles and not transported to the Golgi so that it accumulates as an immature 105-kDa form (26, 27). Indeed, any significant reduction of GPI lipid biosynthesis leads to an increase of the immature 105-kDa form of Gas1p (28). A previous report shows that the ER-to-Golgi transport of Gas1p is blocked in the mcd4Δ–174 mutant at restrictive temperatures, whereas transport of other proteins such as CPY is unaffected (8). As can be seen in Fig. 4A, in mcd4Δ/TbGPI10 cells the mature 125-kDa Gas1p was less abundant than in corresponding wt cells, whereas the immature 105-kDa form was present in normal or increased amounts (see below). Moreover, the mutant cells did not accumulate the typical ER and Golgi proforms of CPY named p1 and p2, respectively, and the mature vacuolar form of CPY was present in normal amounts. The p1 proform of CPY was, however, drastically increased in the sec18 secretion mutant incubated for 1h at 37 °C (not shown). This suggested that the defect of mcd4Δ/TbGPI10 only affects GPI

---

**TABLE 1**

| Sample  | Mature (m) | Immature (i) |
|---------|------------|--------------|
| T1      | 50%        | 50%          |
| T2      | 40%        | 60%          |
| T3      | 30%        | 70%          |
| T4      | 20%        | 80%          |

---

**FIGURE 5.** The percentage of Gas1p that partitions into the aqueous phase is reduced 5-fold during a 20-min incubation in the presence of CHX. A, the relative amounts of immature Gas1p, accumulating in mcd4Δ/TbGPI10 is anchored or not, were partitioned Gas1p before and after PI-PLC treatment in the detergent Triton X-114 (30). Fig. 5B indicates that the percentage of immature Gas1p that partitions into the aqueous phase because of the action of PI-PLC treatment is higher in mcd4Δ/TbGPI10 than in wt cells (62 versus 50%). (It should be noted that it is difficult to shift the total of GPI proteins into the aqueous phase by this technique. In Fig. 5B, even for the mature, GPI-anchored form, only a fraction could be shifted, namely 37% in wt and 42% in mcd4Δ/TbGPI10). Importantly, because the percentages of Gas1p shifted by PI-PLC from detergent to aqueous phase are higher for immature than for mature Gas1p, there is no indication that immature, nonanchored Gas1p would be accumulating, neither in wt nor mcd4Δ/TbGPI10 mutant cells. There are reports showing that during Triton X-114 solubilization at 4 °C, part of GPI-anchored proteins are recovered in the detergent-insoluble pellet. The efficiency of solubilization in our experiment was evaluated by probing equivalent samples of the detergent-insoluble pellet and the detergent extract (Fig. 5C). Although Triton X-114 only solubilized 62 and 80% of Gas1p in wt and mutant cells, respectively, immature Gas1p was solubilized nearly quantitatively in both strains. Thus, the results obtained in Fig. 5 (A and B) are representative for the total of immature Gas1p. Pulse-chase analysis confirmed the severe transport block of Gas1p (Fig. 5D).

To generalize the data obtained with Gas1p, we also investigated the fate of another GPI protein, Yap3p (Yps1p). Yap3p, a 570-amino acid-long protein containing 10 potential N-glycosylation sites was described as a 68-kDa GPI-anchored protein (31), but in other reports using the same antibodies, the immature forms of this protein ran as 80-kDa (32) or as 80- and 100-kDa proteins (33) or as bands at 85 and 115 kDa (34), whereas mature forms ran as a broad smear around 150–200 kDa. As can be seen in Fig. 6A, the mcd4Δ/TbGPI10 mutant (T4) contained much less of the mature (140–240 kDa) Yap3p form than the wt (T2) strain or the

---

**FIGURE 6.** The mcd4Δ/TbGPI10 cells accumulate reduced amounts of mature Gas1p. A, T1–T4 cells and the parental BY4742 cells were cultured in YPDUA at 30 °C, protein extracts were prepared (19) and processed for Western blotting using antibodies against Gas1p and CPY and developed using the LI-COR system. The positions of mature (m), immature (i), and unprocessed precursors (p1 and p2) are indicated. B, cells were grown in YPDUA at 30 °C, media (M) and cell lysates (C) (solubilized with octyl-β-glucoside) were processed for Western blotting using anti-Gas1p antibodies and the ECL system.
gpi1 mutant, but it accumulated an immature 75–100-kDa ER form. Further analysis showed that, similar to Gas1p, Yap3p was secreted from cells into the medium (Fig. 6B). Significantly, the secreted Yap3p consisted mainly of mature Yap3p, whereas in the cell lysate, the mature form of Yap3p was relatively minor. Thus, it seems that the appearance of Yap3p in the culture medium of mcd4/H9004/TbGPI10 cells is not due to cell lysis but rather the secretion of a mature Yap3p form. Secretion of GPI proteins has previously been noted in gpi mutants, e.g. in gpi3 (35). GPI anchoring of Yap3p was tested using PI-PLC and TX-114 partitioning as done for GPI-anchored Gas1p is not efficiently transported from the ER to the Golgi in mcd4/TbGPI10. A, 100 μg/ml cycloheximide (+ CHX) or water (−CHX) were added to exponentially growing MCD4/TbGPI10 (T1) or mcd4/TbGPI10 (T3) cells. After 20 min at 30 °C, the cells were lysed, and proteins were solubilized with TX-114. After sedimenting at 16,000 × g for 5 min to remove insoluble material, the supernatant was warmed to 37 °C, and detergent (D) and aqueous (A) phases were separated and analyzed by Western blotting with anti-Gas1p antibody. The percentage of immature relative to total Gas1p, taking into account the sum of both phases, is indicated (±). B, aliquots of the detergent phases from cells not incubated with CHX were diluted and incubated in the presence (+) or absence (−) of PI-PLC (PLC) to remove anchor lipids (Fig. 1). After incubation the samples were again separated into aqueous (A) and detergent (D) phases and were processed for immunoblotting as above. The percentage of Gas1p present in the aqueous (A) phase compared with the Gas1p in both (A + D) phases is indicated on separate lines for mature (Am) and immature (Ai) forms of Gas1p. C, the insoluble pellet (P) and the soluble supernatant (S) obtained during the initial 16,000 × g spin (see legend for A) were processed for immunoblotting as above. Material corresponding to equivalent amounts of cells was loaded. Blots of A–C were visualized using the LI-COR system. D, cells were labeled with [35S]methionine/cysteine for 12 min and chased for 0 or 40 min. Gas1p was immunoprecipitated and analyzed by immunoprecipitation and autoradiography. Sec18 cells were labeled at 37 °C, where they are unable to transport proteins out of the ER. 

FIGURE 6. Yap3p is not efficiently anchored in mcd4/TbGPI10. A and B, the lysates (A) and media (B) as used for Fig. 4B as well as lysates and media of gpi1 and yap3 prepared in parallel were analyzed for the presence of mature (m) and immature (i) Yap3p by Western blotting. Gpi1Δ cells were grown at 24 °C, incubated for 2 h at 37 °C, and lysed. An unspecific band (u) is pointed out. C, samples analyzed in Fig. 5 (A and B) were also probed with anti-Yap3p antibodies, and the data were quantitated and expressed as in Fig. 5B.

gpi1Δ mutant, but it accumulated an immature 75–100-kDa ER form. Further analysis showed that, similar to Gas1p, Yap3p was secreted from cells into the medium (Fig. 6B). Significantly, the secreted Yap3p consisted mainly of mature Yap3p, whereas in the cell lysate, the mature form of Yap3p was relatively minor. Thus, it seems that the appearance of Yap3p in the culture medium of mcd4/TbGPI10 cells is not due to cell lysis but rather the secretion of a mature Yap3p form. Secretion of GPI proteins has previously been noted in gpi mutants, e.g. in gpi3 (35). GPI anchoring of Yap3p was tested using PI-PLC and TX-114 partitioning as done for...
Gas1p in Fig. 5B. About 60% of the mature Yap3p could be shifted from detergent to aqueous phase by PI-PLC in the wt as well as the mutant (Fig. 6C). Again, the mcd4Δ/TbGPI10 mutant T3 showed a strong accumulation of 75- and 95-kDa ER forms and only 7% of this material could be shifted from the detergent to the aqueous phase by PI-PLC, because the bulk of it partitioned to the aqueous phase even after mock treatment. (It should be noted that in Fig. 6C the amount of immature Yap3p is severely underestimated, because PI-PLC treatment was done with the Triton X-114 detergent phase, and the bulk of the immature Yap3p had already been lost into the aqueous phase of the initial phase separation done to produce this detergent phase that was then treated with PI-PLC). Low amounts of the same 75- and 95-kDa ER forms were visible in the detergent phase of wt cells and were efficiently shifted from the detergent to the aqueous phase by PI-PLC (Fig. 6C, not quantitated). The region <65 kDa contains nonspecific bands. During CHX treatment, the 75–95-kDa ER forms vanished in wt cells, but not in mcd4Δ/TbGPI10 cells (not shown). A control analogous to Fig. 5C showed that Triton X-114 had quantitatively extracted Yap3p form T3, whereas a small percentage of mature Yap3p of T1 remained in the pellet (not shown). We interpret these results as to mean that Yap3p, quite in contrast to Gas1p, fails to be anchored efficiently in mcd4Δ/TbGPI10 and accumulates in an unanchored form in the ER. This interpretation is based on the observation that nonanchored Gas1p partitions into the aqueous phase during Triton X-114 phase separation (30, 36) and the assumption that the same may hold for unanchored Yap3p. To summarize, in mcd4Δ/TbGPI10 cells, Yap3p accumulates in the ER, a small amount is anchored and remains cellular, and a larger part travels through the Golgi and ends up in the medium. It is presently unclear whether that latter material is first anchored but secreted because its structurally abnormal anchor is hydrolyzed at the cell surface, or whether this material is transported through the Golgi in an unanchored form.

The mcd4Δ/TbGPI10 Cells Do Not Introduce Ceramides into Their GPI Anchors in the ER—The mature GPI proteins contain a different lipid moiety than the primary GPI lipid, which is attached to them after they have entered the ER. This primary GPI lipid probably contains the C16 and C18 fatty acids typically found in phosphatidylinositol in yeast (37). Most mature GPI proteins of yeast contain a ceramide moiety, whereas a minor fraction contains a modified diacylglycerol containing C26:0 in sn2 (34, 37, 38). Thus, all mature GPI proteins of yeast are endowed with lipid moieties containing C26 or hydroxylated C26 fatty acids, and the mature lipid moieties are introduced by a remodeling step that replaces the primary lipid moiety. As shown in Fig. 7 (lanes 5–7), T1 and T2 MCD4/TbGPI10 cells make a normal set of anchor lipids, namely pG1, a C26:0-containing PI species migrating slightly faster than the abundant PI present in the lipid extract of wt cells (lane 4), and inositolphosphorylceramides B and C, containing PHS-C26:0 and PHS-C26:0-OH, respectively. In contrast, T3 and T4 mcd4Δ/TbGPI10 cells make only pG1, but no inositolphosphorylceramides (Fig. 7, lanes 8 and 9). Their ceramide biosynthesis however must be normal because they make a normal set of sphingolipids (Fig. 7, lanes 1–3), although in lower than normal quantities. This probably is a consequence of the slower growth rate and a concomitant slowing of the transfer of [3H]inositol phosphate from PI to ceramide.

Cell Wall Biogenesis and Bud Site Selection Are Defective in mcd4Δ/TbGPI10 Cells—Many mutations in the GPI biosynthesis pathway cause cell wall fragility, for which the cell tries to compensate by inducing chitin biosynthesis, and cells therefore become hypersensitive to calcofluor white, a chitin-binding toxic compound. mcd4 mutants being affected in GPI biosynthesis not unexpectedly display osmotic sensitivity and lysis in hypotonic media, increased sensitivity to calcofluor white, SDS, zymolyase, caffeine, and staurosporine, an inhibitor of Pkc1p (17, 39). As shown in Fig. 8 (A and B), mcd4Δ/TbGPI10 cells were hypersensitive to calcofluor white, caffeine, and neomycin and also were unable to survive a sudden heat shock (Fig. 8C). Despite these several signs of cell wall fragility, mcd4Δ/TbGPI10 cells did not display any hypersensitivity to SDS and were not hypersensitive to staurosporine (not shown), suggesting that the cells are osmotically competent. Thus, certain symptoms of cell wall fragility are present in mcd4Δ/TbGPI10 cells but others, typically present in mcd4 mutants, are absent. The mcd4Δ/TbGPI10 cells also were Cu2+-hypersensitive (Fig. 8B), whereas certain mcd4 alleles had been shown to be copper-resistant (15).

Compared with wt cells, the mcd4Δ/TbGPI10 cells are unusually large and grow in small clumps of round cells (not shown). A similar phenotype of abnormally large and round cells, growing in small clumps because of a cell separation defect, has been described in a mcd4 mutant and has been attributed to its inability to polarize its secretion apparatus (14, 17). Microscopic inspection of haploid mcd4Δ/TbGPI10 cells after calcofluor white staining indicated that cells have only slightly more chitin in their cell walls than wt cells but that they
have a defect in axial bud site selection. Many cells, instead of the normal axial, showed a bipolar or unipolar or random bud site selection pattern (Fig. 9A). The same bud site selection defect was equally seen when cells were grown in minimal medium, but in this medium they also had more chitin all over their cell walls (not shown). As a positive control we used elo3Δ cells, which do not show the normal axial pattern but exhibit a mix of bipolar, unipolar, and random bud site selection patterns (Fig. 9B) (40). Transfection of mcd4Δ/Tbgp110 with a plasmid harboring the wt MCD4 gene abolished the bud site selection defect, because buds faithfully emerged next to the previous bud emergence site (Fig. 9A).

The same bud selection defect was also found in the mcd4Δ/mcd4ts strain grown at a semi-permissive temperature (32° and 34 °C) (Fig. 9B). These mutant cells additionally exhibited a deregulated bud emergence generating multibudded cells (Fig. 9B), a phenomenon that was only very occasionally observed in mcd4Δ/Tbgp110. The gpi7Δ and gpi1 mutants (Fig. 1), lacking an EtN-P on Man2 or being unable to make GPI anchors at elevated temperatures (41) did not exhibit any bud site selection defect (Fig. 9B), although gpi1 mutants generate multibudded cells (41). These findings suggest that bud site selection is not just dependent on the synthesis of sufficient amounts of GPI proteins but specifically depends on the EtN-P on Man1.

**DISCUSSION**

Our report shows that the essentiality of MCD4 in yeast can be suppressed by the presence of *T. brucei* Gpi10, an enzyme that allows the completion of GPI lipids beyond the stage of the Man2-GlcN-acyl-PI intermediate that has been shown to accumulate in mcd4Δ mutants (7). This reconfirms the previously postulated importance of the EtN-P side chain on Man1 for yeast Gpi10p and the correctness of the idea that MCD4 is required for addition of Man3 because it allows the biosynthesis of the correct substrate for Gpi10p (9, 11). The resulting mcd4Δ/Tbgp110 yeast strain grows more slowly than wt and shows various abnormalities in the biosynthesis and transport of GPI proteins, but it would appear that neither the enzymes required to transform the Man3-GlcN-acyl-PI into a mature GPI nor the transamidase have an absolute requirement for a EtN-P on Man1 (Fig. 1). In contrast to yeast, the human MCD4 ortholog PIG-N is not required for the survival of tumor cell lines in vitro (9). In vivo metabolic labeling with [3H]mannose of PIG-N knock-out (KO) cells shows that the GPI biosynthesis stops at H7’ (mammalian equivalent of M4*) instead of H8 (mammalian equivalent of CP2), but quantitatively there is not more H7’ in PIG-N KO cells than there is H8 in wt cells, whereas transamidase mutants such as the hGA11 KO cells accumulate massive amounts of H8 and of earlier GPI intermediates (9). Although these KO tumor cell lines may be genetically unstable and therefore not truly comparable among each other, one naively may interpret the above findings to mean that the human transamidase uses GPI lipids lacking EtN-P on Man1 quite efficiently, or else that the transamidase is absolutely unstable and therefore not truly comparable among each other. Thus, we believe that the transamidase is absolutely not rate-limiting in the mammalian system. In contrast, the accumulation of M4* in mcd4Δ/Tbgp110 cells (Fig. 3A) suggests that yeast transamidase does not work efficiently with GPI lipids lacking EtN-P on Man1, or else that the GPI transamidase of mcd4Δ/Tbgp110 cells is blocked by anchored GPI proteins, which accumulate because they are not efficiently transported to the Golgi. These two possibilities are not mutually exclusive. The latter idea is, however, less likely, because no accumulation of CP2 can be observed in sec18 at 37 °C, which accumulate GPI-anchored proteins in the ER because of a secretion block (42). Thus, we believe that the transamidase is working more slowly in mcd4Δ/Tbgp110 because M4* is not its optimal substrate. This is also supported by the finding that the amount of [3H]inositol-labeled proteins is severely reduced (Fig. 3D) and that certain GPI proteins accumulate as unanchored ER forms. Intriguingly, this was only observed with Yap3p, whereas Gas1p was efficiently anchored. How can we explain this different fate of Gas1p and Yap3p?
The quality of the GPI attachment signal and hence the probability that a protein will be GPI-anchored can be calculated using the algorithms elaborated by Birgit Eisenhaber and co-workers (mendel.imp.univie.ac.at/home/Birgit.Eisenhaber/index.html). The predictor for fungi gives GPI anchoring probability scores between 5 and 20 for most of the ascertained GPI proteins of yeast, a score of 13.7 for Gas1p, but there is a score of only 0.65 for Yap3p. Nevertheless, data in Fig. 6C indicate that in wt cells (T1) at least 59% of Yap3p are GPI-anchored, confirming an earlier report by others (31). The available microsomal assays for the GPI transamidase allowed to establish that the Gpi8p protease attacks the ω residue, thereby allowing the C-terminal anchor attachment signal to leave and forming a covalent acyl intermediate, which in turn is attacked by the GPI lipid to release the enzyme. It is conceivable that the binding of GPI lipid and protein substrate to the enzyme is cooperative and that low score GPI proteins such as Yap3p are more dependent on this cooperative effect for efficient anchoring than high score proteins such as Gas1p. Thus, we speculate that in mcd4Δ/TbGPI10 cells the lack of appropriate GPI lipids may lead to a deficiency of GPI anchoring, which specifically affects low score proteins.

In yeast, the reduced surface expression of GPI proteins in mcd4Δ/TbGPI10 can be traced to several reasons, depending on the protein; Gas1p seems to be inefficiently transported and, in addition, to be lost into the medium, and Yap3p also is lost from the surface and additionally is not anchored efficiently. The analysis of the lipid anchor of Gas1p only identified fatty acids but no ceramide (38); thus, the lack of ceramide remodeling cannot directly be responsible for the failure of mcd4Δ/TbGPI10 cells to transport Gas1p from the ER to the Golgi. One possibility is that EtN-P on Man1 is required for the interaction of Gas1p with the cargo receptor Emp24p, which was shown to physically interact with Gas1p (43).

FIGURE 9. mcd4Δ/TbGPI10 cells have a bud site selection defect. A and B, cells were kept in continuous culture at 30 °C during 10 generations and stained with calcofluor white and photographed. The prevalent types of scar patterns are shown. The mcd4Δ cells were grown at semipermissive temperatures (32 or 34 °C). The gpi1 mutant grows at 30 °C but is unable to grow at 32 °C.

The complete absence of ceramide anchors in mcd4Δ/TbGPI10 cells suggests that, similar to GPI10, the hypothetical ceramide remodelase requires the presence of EtN-P on Man1. Alternatively, it also may be that, beyond being the EtN-P transferase, MCD4 also acts as a ceramide remodelase, capable of exchanging diacylglycerol for ceramide. Indeed EtN-P transferase and remodelase reactions are similar in that both represent transterification reactions involving phosphodiester linkages. The possibility that Mcd4p acts as ceramide remodelase is also supported by the fact that the deletion of GPI7 (Fig. 1) leads to a specific defect of ceramide remodeling in the Golgi (23). This possibility is presently further investigated.

MCD4 mutants were isolated in many different contexts and found to be deficient in many apparently unrelated biological functions. Because the mcd4Δ/TbGPI10 cells not only lack EtN-P on Man1 of the GPI-anchored proteins but also exhibit a severe reduction of many GPI proteins on their surface, we tried to compare their phenotype with the ones of the few other viable gpi mutants hoping to identify some characteristics that may give us a hint about a more specific function of the EtN-P on Man1 and the introduction of ceramide into the GPI anchors in the ER. Such a specific function was recently suggested for the EtN-P on Man2, added by Gpi7p: Lack of EtN-P on Man2 leads to a mis-sorting of the daughter cell specific, GPI-anchored endoglucanase Egt2p (44). We started to investigate this phenotype in mcd4Δ/TbGPI10 but abandoned the investigation because >90% of the HA-tagged Egt2p was secreted by both wt (T1) and mcd4Δ/TbGPI10 (T3) in the form of a 65-kDa fragment (reducing SDS-PAGE) rather than the expected >100-kDa form (not shown).

We find a distinct bud site selection defect in all mcd4Δ/TbGPI10 cells. Although many genes are known to affect the bipolar bud site selection in diploid cells, much fewer genes are known to affect the normal axial budding pattern of haploid cells, and the two classes of genes are largely nonoverlapping (45). Of the few genes related to GPI protein expression, the deletion of GAS1, Ccw12, Bst1, and GPI7 affect only bipolar bud site selection in diploids but were found to be without any effect on budding in haploid cells (40). Obviously, if GPI anchoring were required for bud site selection, this may easily have been overlooked, because most deletion mutants in the GPI anchor biosynthesis pathway of yeast are lethal. Here we report on a bud site selection defect of mcd4Δ/TbGPI10. This finding suggests that some GPI-anchored protein(s) may be important for bud site selection in haploids. At the same time our data show that haploid gpi7Δ and gpi1Δ and bst1Δ mutants do not exhibit a similar defect (Fig. 9B) (40). Further work is necessary to firmly establish and understand the specific role of the EtN-P side chain on Man1 in axial bud site selection.
Acknowledgments—We thank Howard Riezman, Taroh Kinoshita, Niahm Cawley, Yoshifumi Jigami, Dennis Voelker, Sabine Strahl, and Peter Orlean for strains, plasmids, and reagents.

REFERENCES

1. Ferguson, M. A. J., Kinoshita, T., and Hart, G. W. (2006) in *Essential Glycobiology* (Varki, A., Bottozzi, C., Cummings, R., Etzler, M., Esko, J., Freeze, H., Hart, G., and Stanley, P., eds) 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

2. Kinoshita, T., and Inoue, N. (2000) *Curr. Opin. Chem. Biol.* 4, 632–638

3. Ferguson, M. A., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1998) *Science* 239, 753–759

4. McConville, M. J., Collridge, T. A., Ferguson, M. A., and Schneider, P. (1993) *J. Biol. Chem.* 268, 15595–15604

5. Homans, S. W., Ferguson, M. A., Dwek, R. A., Rademacher, T. W., Anand, R., and Williams, A. F. (1988) *Nature* 333, 269–272

6. Sutterlin, C., Horvath, A., Gerold, P., Schwarz, R. T., Wang, Y., Dreyfuss, M., and Riezman, H. (1997) *EMBO J.* 16, 6374–6383

7. Imhof, I., Flury, I., Vionnet, C., Roubaty, C., Egger, D., and Conzelmann, A. (2004) *J. Biol. Chem.* 279, 19614–19627

8. Gaynor, E. C., Mondesert, G., Grimme, S. J., Reed, S. I., Orlean, P., and Hart, G. W. (2006) in *Cell Biology and Dynamics of Yeast* (Daum, G., ed). *J. Biol. Chem.* 271, 31869–31879

9. Hong, Y., Maeda, Y., Watanabe, R., Ohishi, K., Mishkind, M., Riezman, H., and Kinoshita, T. (1999) *J. Biol. Chem.* 274, 35099–35106

10. Imhof, I., Canivenc-Gansel, E., Meyer, U., and Conzelmann, A. (2000) *Glycobiology* 10, 1271–1275

11. Sutterlin, C., Escribano, M. V., Gerold, P., Maeda, Y., Mazon, M. J., Kinoshita, T., Schwarz, R. T., and Riezman, H. (1998) *Biochem. J.* 332, 153–159

12. Storey, M. K., Wu, W. I., and Voelker, D. R. (2001) *Biochim. Biophys. Acta* 1532, 234–247

13. Toh-e, A., and Oguchi, T. (2002) *Genes Genet. Syst.* 77, 309–322

14. Mondesert, G., Clarke, D. J., and Reed, S. I. (1997) *Genetics* 147, 421–434

15. Toh-e, A., and Oguchi, T. (2001) *Genes Genet. Syst.* 76, 393–410

16. Nagamune, K., Nozaki, T., Maeda, Y., Ohishi, K., Fukushima, T., Hara, T., Schwarz, R. T., Sutterlin, C., Brun, R., Riezman, H., and Kinoshita, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 10336–10341

17. Packeiser, A. N., Urakov, V. N., Polyakova, Y. A., Shimanova, N. I., Scherbukhin, V. D., Smirnov, V. N., and Ter-Avanesyan, M. D. (1999) *Yeast* 15, 1485–1501

18. Sherman, F. (2002) *Methods Enzymol.* 350, 3–41

19. Kushnirov, V. V. (2000) *Yeast* 16, 857–860

20. Guillans, I., Pfefferli, M., and Conzelmann, A. (2000) *Methods Enzymol.* 312, 506–515

21. Mumberg, D., Muller, R., and Funk, M. (1994) *Nucleic Acids Res.* 22, 5767–5768

22. Maneesri, J., Azuma, M., Sakai, Y., Igarashi, K., Matsumoto, T., Fukuda, H., Kondo, A., and Ooshima, H. (2005) *J. Biochem.* 99, 354–360

23. Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A., and Benghezal, M. (1999) *J. Biol. Chem.* 274, 15251–15261

24. Flury, I., Benachour, A., and Conzelmann, A. (2000) *J. Biol. Chem.* 275, 24458–24465

25. Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A., and Benachour, A. (1998) *Glycobiology* 8, 761–770

26. Niu, L., Horvath, A., and Riezman, H. (1993) *J. Biol. Chem.* 268, 10558–10563

27. Doering, T. L., and Schekman, R. (1996) *EMBO J.* 15, 182–191

28. Benghezal, M., Lipke, P. N., and Conzelmann, A. (1995) *J. Cell Biol.* 130, 1333–1344

29. Conzelmann, A. (2005) (Daun, G., ed) *Cell Biology and Dynamics of Yeast Lipids*, pp. 133–159, Research Signpost, Trivandrum, Kerala, India

30. Watanabe, R., Funato, K., Venkataraman, K., Futereman, A. H., and Riezman, H. (2002) *J. Biol. Chem.* 277, 49538–49544

31. Ash, J., Domínguez, M., Bergeron, J. J., Thomas, D. Y., and Bourbonnais, Y. (1995) *J. Biol. Chem.* 270, 20847–20854

32. Tomishige, N., Noda, Y., Adachi, H., Shimoi, H., and Yoda, K. (2005) *Yeast* 22, 141–155

33. Barz, W. P., and Walter, P. (1999) *Mol. Biol. Cell* 10, 1043–1059

34. Reggiori, F., Canivenc-Gansel, E., and Conzelmann, A. (1997) *EMBO J.* 16, 3506–3518

35. Vossen, J. H., Muller, W. H., Lipke, P. N., and Klis, F. M. (1997) *J. Bacteriol.* 179, 2202–2209

36. Sobering, A. K., Watanabe, R., Romeo, M. J., Yan, B. C., Specht, C. A., Orlean, P., Riezman, H., and Levin, D. E. (2004) *Cell* 117, 637–648

37. Sipos, G., Reggiori, F., Vionnet, C., and Conzelmann, A. (1997) *EMBO J.* 16, 3494–3505

38. Fankhauser, C., Homans, S. W., Thomas-Oates, J. E., McConville, M. J., Desponds, C., Conzelmann, A., and Ferguson, M. A. (1993) *J. Biol. Chem.* 268, 26365–26374

39. Kaptyn, I. C., Ram, A. F., Groos, E. M., Kollar, R., Montijn, R. C., Van, D. E. H., Llobell, A., Cabib, E., and Klis, F. M. (1997) *J. Bacteriol.* 179, 6279–6284

40. Ni, L., and Snyder, M. (2001) *Mol. Biol. Cell* 12, 2147–2170

41. Leidich, S. D., and Orlean, P. (1996) *J. Biol. Chem.* 271, 27829–27837

42. Sipos, G., Puoti, A., and Conzelmann, A. (1994) *EMBO J.* 13, 2789–2796

43. Muniz, M., Nuoffer, C., Hauri, H. P., and Riezman, H. (2000) *J. Cell Biol.* 148, 925–930

44. Fujita, M., Yoko-O, T., Okamoto, M., and Jigami, Y. (2004) *J. Biol. Chem.* 279, 51869–51879

45. Casamayor, A., and Snyder, M. (2002) *Curr. Opin. Microbiol.* 5, 179–186
Ethanalaminephosphate Side Chain Added to Glycosylphosphatidylinositol (GPI) Anchor by Mcd4p Is Required for Ceramide Remodeling and Forward Transport of GPI Proteins from Endoplasmic Reticulum to Golgi
Yonghua Zhu, Christine Vionnet and Andreas Conzelmann

J. Biol. Chem. 2006, 281:19830-19839.
doi: 10.1074/jbc.M601425200 originally published online May 16, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M601425200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 25 of which can be accessed free at
http://www.jbc.org/content/281/29/19830.full.html#ref-list-1