Glycosylphosphatidylinositol (GPI) Proteins of Saccharomyces cerevisiae Contain Ethanolamine Phosphate Groups on the α1,4-linked Mannose of the GPI Anchor

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In humans and Saccharomyces cerevisiae the free glycosylphosphatidylinositol (GPI) lipid precursor contains several ethanolamine phosphate side chains, but these side chains had been found on the protein-bound GPI anchors only in humans, not yeast. Here we confirm that the ethanolamine phosphate side chain added by Mcd4p to the first mannose is a prerequisite for the addition of the third mannose to the GPI precursor lipid and demonstrate that, contrary to an earlier report, an ethanolamine phosphate can equally be found on the majority of yeast GPI protein anchors. Curiously, the stability of this substituent during preparation of anchors is much greater in gpi7Δ sec18 double mutants than in either single mutant or wild type cells, indicating that the lack of a substituent on the second mannose (caused by the deletion of GPI7) influences the stability of the one on the first mannose. The phosphodiester-linked substituent on the second mannose, probably a further ethanolamine phosphate, is added to GPI lipids by endoplasmic reticulum-derived microsomes in vitro, but cannot be detected on GPI proteins of wild type cells and undergoes spontaneous hydrolysis in saline. Genetic manipulations to increase phosphatidyethanolamine levels in gpi7Δ cells by overexpression of PSD1 restore cell growth at 37 °C without restoring the addition of a substituent to Man2. The three putative ethanolamine-phosphate transferases Gpi13p, Gpi7p, and Mcd4p cannot replace each other even when overexpressed. Various models trying to explain how Gpi7p, a plasma membrane protein, directs the addition of ethanolamine phosphate to mannose 2 of the GPI core have been formulated and put to the test.

Many glycoproteins of lower and higher eukaryotes are attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI)1 (1, 2). The carbohydrate structure linking the C-terminal end of GPI proteins to the lipid moiety is identical in GPI anchors from all organisms analyzed so far, namely protein-CO-NH-(CH2)-PO4-6Man/H9251.

1 The abbreviations used are: GPI, glycosylphosphatidylinositol; CP, complete precursor; Chx, cycloheximide; EtN, ethanolamine; EtN-P, ethanolamine phosphate; Ins, myo-inositol; JBAM, jack bean α-mannosidase; HF, hydrofluoric acid; Man, mannose; MIPC, mannosyl-IPC; MIPrC, inositolphosphoryl-MIPC; PE, phosphatidylethanolamine; PI, phosphatidylinositol; WT, wild type; ER, endoplasmic reticulum; PLC, phospholipase C; UPR, unfolded protein response; ConA, concanavalin A.

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biosynthetic process remain to be elucidated. One particular difficulty is to know which GPI lipid is attached to nascent proteins by the GPI transamidase complex in the ER. Even though there is a wealth of partial structures of lipids accumulating in mutants that are unable to synthesize or to attach GPI lipids to proteins, or of lipids that are synthesized by microsomes in vitro, it is doubtful that all these lipids are normal intermediates of the pathway. Thus, it cannot be excluded a priori that under physiological conditions certain Etn-Ps are added to GPI anchors only after the GPI lipid has been added to proteins or the protein has left the ER. Here we describe our attempts to address a few of the unresolved questions about the biosynthetic route and the role of the Etn-P residues on Man1 and Man2 in yeast.

EXPERIMENTAL PROCEDURES

Strains, Media, and Materials—Saccharomyces strains are listed in Table I. Cells were grown on rich medium (YPD) or minimal media SDaaUA or SGaaUA, containing 2% glucose (D) or galactose (G) at 30 °C and amino acids (designated with aa), uracil (U), and adenine (A) but without inositol (Ina) (25). Chemicals, radiochemicals, and inhibitors were from sources described (11). PI-specific PLC (PI-PLC) from Bacillus cereus was from ICN Biomedicals (number 195685) (Aura- rora, OH) or Roche Molecular Biochemicals (number 1-143-060) (Rot- kreuz, Switzerland); GPI-PLD purified from bovine serum was the kind gift of Dr. U. Brodbreck. Pentoxifylline, dipyramidole, and ethaverine were from Sigma, 3-isobutyl-1-methylxanthine and papaverine were from Fluka, Buchs, Switzerland. Pronase for anchor preparation was from Sigma, catalog number P-5147, or Roche Molecular Biochemicals, (incorporating number 165-921. Concanavalin (ConA)-Sepharose was from Amersham Biosciences (number 17-0440-01).

Preparation of Radiolabeled GPI Protein Anchor Peptides and Anchor Head Group Peptides—Exponentially growing cells were labeled with myo-[2-3H]inositol in Ina-free SDaaUA as described (26). Washed cells were broken with glass beads in chloroform/methanol/water (10:10:3), and then Lester solvent (ethanol/water/diethyl ether/pyridine/concentrated NH4OH (15:15:5:1:0.018)) at 37 °C for 15 min as described (26, 27). In some experiments (Table IV) the latter solvent was replaced by chloroform, methanol, 1.5 mm triethylamine (10:10:3) or proteins were extracted without preliminary organic solvent extraction (Table V) by just boiling cells in sample buffer, or by breaking cells with glass beads in TPIN buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 30 μg/ml leupeptin, pepstatin, and antipain) or by incubation for 5 min at room temperature in 100 mM NaOH as described (28). Proteins were solubilized by boiling in sample buffer K (60 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% 2-mercaptoethanol, 0.005% bromphenol blue). Anchor peptides were then prepared essentially as outlined in Fig. 3 and described before (26) except that the number of delipidation and washing steps was reduced to obtain quantitative recovery rather than full delipidation of anchor peptides and the ConA-Sepharose buffer was 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM each of CaCl2, MgCl2, MnCl2, phenylmethylsulfonyl fluoride, and benzamidine. Soluble head groups were obtained from purified radiolabeled anchor peptides through limited methanolic NH4 deacylation (29) followed by PI-PLC treatment, for which the peptides were dissolved in 20 mM Tris-HCl, pH 7.5, 0.2% EDTA, 20% 1-propanol or by GPI-PLD treatment in 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 20% propanol, 2.6 mM CaCl2. Incubations with PI-PLC or GPI-PLD were for 16 h at 37 °C. Lipids were removed by butanol extraction.

Testing Enzymes for Contaminating Phosphodiesterase Activity Removing Etn-P Side Chains—Radiolabeled CP lipids were generated by metabolic labeling of pww40 with [3H]mannose (30) or sec18 gus8 double mutants with myo-[2-3H]Ins. CP lipids were purified by preparative TLC and then incubated under exactly the same conditions as used for preparing GPI anchor peptides. In some experiments we added a mixture of the phosphodiesterase inhibitors pentoxifylline, dipyramidole, ethaverine, 3-isobutyl-1-methylxanthine and papaverine at 1, 0.25, 0.1, and 0.25 μM final concentrations, respectively. To test for a phosphodiesterase activity that would remove Etn-P, CaCl2 in GPI-PLD buffer was replaced by 10 mM EDTA so that the GPI-PLD itself was inactive (31). After incubation the potential degradation of CP lipids and the appearance of less polar lipids was assessed by TLC in solvent 1 followed by radioscopic/fluorography.

Analysis of Head Groups—Labeled head groups were subjected to analysis by HPLC and radiochromatography. N-acetylation by methods listed in Ref. 11, and paper chromatography in solvent methyl ethyl ketone/pyridine/water (20:12:11) (9). myo-[4C]Ins was added to each sample as an internal standard before paper chromatography allowing for exact positioning of Man-GlcNAc-Ins and GlcNAc-Ins peaks.

Lipid Analysis—Lipids were extracted from labeled cells using CHCl3/CH3OH/H2O (10:3), desalted by butanol/water partitioning, and analyzed by TLC on Silica Gel 60 plates using the same solvent (solvent 1) followed by fluorography.

Biosynthesis of GPI Lipids in Vitro—For GPI biosynthesis in microsomes in vitro we followed a previously used protocol (11). Briefly, spheroplasts were generated by incubation for 60 min at 37 °C in buffer A (10 mM azide, 1.4 M sorbitol, 50 mM K2HPO4, pH 7.5, 40 mM 2-mercaptoethanol) using Zymolyase (0.2 mg/ml) or Quantzyme (3 units/1 A260 unit of cells), spheroplasts were washed 2 times in the same buffer but without 2-mercaptoethanol, were broken by forcing them through a 0.4-mm needle using a syringe, the cell lysate was centrifuged at 4 °C for 3000 × g for 5 min, and then 75,000 × g for 60 min. Pellets F5 and P75 were resuspended in 0.8 μM sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml chymostatin, 2 μg/ml antipain and could be frozen at that stage. For the standard assay the two pellets were pooled, diluted into 100 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 0.5 mM MnCl2, 1 mM EGTA, 1 mM ATP, 1 mM CoA, 1 mM GDP-mannose, 20 μM MgCl2, 50 μM unlabeled myo-[3H]mannose, and 50 μg/ml nucleasein and incubated in a final volume of 100 μl with 3–6 μCi of UDP-[3H]GlCNac for 60–90 min at 30 °C.

Plasmids and Plasmid Construction—A pUPRE expressing lacZ from an artificial promoter containing UPRE was obtained from Dr. Ralph Menzel, Berlin (32). For Fig. 1A we used multicopy TOp2141, TOP662, or YEp24 containing PSD1, ECOM3, or no insert that all were contributed by Dr. Akio Toh-e (33). pBF11 containing GPI7 with a deletion of amino acids 192–321 was constructed by opening pBP43 (YCP33 containing GPI7 behind its own promoter) with SmaI and BssHI. The thus generated gap was filled with a Smal-BssHI fragment from pBF43. pBF113 was constructed by amplifying the N-terminal part of G1p7 by PCR using primers 5′-agaagctcacaagatagatggtgtcagtattg-3′ and 5′-ggcagCGCGGTAACCGAGTCGTTT-3′ having restriction sites MluI and AgeI (uppercase). The last 39 amino acids of Wbp1p (including its transmembrane domain and the KXXK motif), the
stop codon plus the transcription terminator were amplified with primers 5′-cggacACCGGTACCGTTTggttagttatagcge ccatt-3′ and 5′-cggac-
gaatgacgtcGGTACCaccttattat-3′. The two PCR fragments were cut with MluI and EcoRV and MluI and KpnI, respectively, and ligation into pBF43 opened with EcoRV and KpnI. pBF114 was constructed by amplifying nucleotides 409–1197 of GPI7 with primers 5′-tggTGATCCAGGtttctattatagcgtgaccttattat-3′ and 5′-ggtTGAACCGGTACCGTTTTggttaggtttagttagcc-3′ and the transcription terminator of GPI7 with primers 5′-cggacGAA-
gaatagctcGGTACCaccttattat-3′ and 5′-cggacCAGGTTTggttaggggtaggtttagttagcc-3′. The two PCR fragments were used as templates for crossed PCR and coding RSKKHQ in uppercase) and 5′-aggtgactagctcGGTACCaccttattat-3′ obtained by amplifying the C terminus of Gpi7p with primers 5′-TCTA-
gtgagtacatGGTACCaccttattat-3′. The two PCR fragments were used as templates for crossed PCR and the final product was cut with BssHII and KpnI and then ligated into pBF43 that was opened in the same way. The multicopy version of the intermediates accumulating in mcd4 mutants. Several mcd4 mutants had previously been labeled with [3H]inositol but accumulated only very small amounts of Ins-labeled GPI lipids that could not be structurally analyzed (15, 19, 37).

We therefore decided to analyze the in vitro GPI biosynthesis in yeast strains carrying either a temperature-sensitive mutation in MCD4/SUSU21 (38) or the wild-type MCD4 gene under control of the GAL1 promoter (19). Microsomes from a wild-type strain make the complete GPI precursor CP2 irrespective of the carbon source on which cells have been grown (Fig. 2, lanes 1 and 2). In contrast, Mcd4p-depleted microsomes accumulate a GPI intermediate termed lipid 4c (Fig. 2, lane 4) and are still able to make CP2, although considerably less than wild type. In microsomes from a temperature-sensitive mcd4, a stronger block of GPI biosynthesis is observed (Fig. 2, lane 5), and neither M4 nor CP2 is made. Lipid 4c is less polar than lipid 031b (Man-Man-Man-[EtN-P]→Man-GlcN-(acyl)→P1), suggesting that it is an earlier intermediate in GPI biosynthesis. Structural characterization of lipid 4c shows that lipid 4c contains a Manα1-GlcN-Ins core structure and that both mannoses can be removed by treatment with JBAM (Fig. 2B). A similar workup of the total pool of GPI lipids generated by mcd4–174 microsomes showed that none of them had more than two mannoses (not shown). This finding is in agreement with the idea that in yeast, differently than in mammals, the addition of EtN-P is a prerequisite for the addition of Man3 as proposed before (16, 35). It is still possible that lipids 3-1-2 and 11-2 of lipid 4c fail to detect any substituent on Man1 of protein anchors we decided to reinvestigate this issue. Indeed, in our previous report we prepared anchors in large quantities for analysis by

**RESULTS**

**Addition of Ethanolamine Phosphate to Man1** Is a Prerequisite for the Addition of Man3 by Gpi10p—During GPI biosynthesis, Man3 is transferred from dolicholphosphomannose to Man2 by PIG-B in mammals and its homologue GPI10 in yeast (11, 34, 35). Mammalian and yeast mutants in these genes accumulate Man−[EtN-P]→Man-GlcN-(acyl)→P1. When treated with the fungal inhibitor YW3548, yeast cells accumulate Man-Man-GlcN-(acyl)→P1 and YW3548 has therefore been postulated to be an inhibitor of the EtN-P transferase PIG-N/MCD4 (35, 36) than of Gpi10p, as initially proposed. This latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive, and that this latter pathway cannot provide substrates for the GPI transamidase that would allow cells to survive. It is still possible that lipids 3-1-2 and 11-2 of lipid 4c fail to detect any substituent on Man1 of protein anchors we decided to reinvestigate this issue. Indeed, in our previous report we prepared anchors in large quantities for analysis by
GC-MS, NMR, exoglycosidase sequencing, and fast atom bombardment mass spectrometry on the basis of their biochemical and biophysical properties and without the preliminary purification of any particular GPI protein (14). The methods used previously for purification of GPI anchors might have led to the loss of phosphodiester-linked substituents because some endogenous phosphodiesterases might have survived the initial high pH treatment, pH 11, during glass bead disruption of cells and been active during subsequent membrane preparation steps. Also, the enzymes utilized for the further isolation and purification of the hydrophilic GPI anchor head groups such as Pronase, endoglycosidase H, and PI-PLC might have been contaminated by phosphodiesterases. We thus decided to take a different approach, i.e., to use [3H]Ins-labeled cells as starting material and to follow the purification scheme depicted in Fig. 3. We reasoned that the initial denaturation in organic solvent and the subsequent boiling in SDS ought to rapidly and efficiently inactivate any untoward hydrolases. Moreover, we took care to screen all enzymes as well as ConA-Sepharose for the presence of contaminating phosphodiesterases by incubating purified radiolabeled CP lipids under the same conditions as used for preparing GPI anchor head groups. After incubation, the status of the radiolabeled CP was assessed by TLC and radioscanning/fluorography. As can be seen in Fig. 4, only minor quantities of more hydrophobic lipids running in the region of M4 were generated through 16-h incubations with purified bovine GPI-PLD, and the brands of PI-PLC of B. cereus or Pronase specified under "Experimental Procedures." The appearance of small amounts of material running in the region of M4 could not be prevented by phosphodiesterase inhibitors. When testing for phosphodiesterase activity in ConA-Sepharose we found that affinity chromatography degrades CP lipids and transforms them into a lipid comigrating with M4 as summarized in Table II. It appeared that degradation was not caused by ConA-Sepharose but largely by the buffer. Divalent cations were only partially responsible for the degradation and about 10% of degradation occurred even in their absence. Interestingly, degradation was strongly enhanced by NaCl. As the main degradation product comigrates with M4/1, the lipid that accumulates in gpi7 mutants and contains an EtN-P on Man1 but lacks a substituent on Man2 (12) (not shown), we surmise that the ConA buffer removes a labile substituent from

![Fig. 2. Cells deficient in MCD4 accumulate GPI intermediates in vitro.](http://www.jbc.org/)

A. Before being converted to spheroplasts, FBY413 (wild-type, lanes 1 and 2) and FBY1104 (mcd4::HIS3 GAL1::MCD4, lanes 3 and 4) were grown for 16 h on glucose or galactose at 30 °C for depletion of Mcd4p, 521-17A-H42 (ssa2::mcd4Δ, lane 5) and 17A-H42 (corresponding wild-type, lane 6) were grown at 24 °C and then for 2 h at 37 °C. Microsomes were prepared and incubated with UDP-[^3H]GlcNAc, GDP-Man, tunicamycin, CoA, and ATP at the indicated temperatures for 1 h. Lane 7, contains a lipid extract of gpi7Δ cells labeled with [3H]Ins. Lipids were extracted, desalted, and analysed by TLC using solvent 1 and fluorography. B. Microsomes from strain 521-17A-H42 were labeled with UDP[^3H]GlcNAc and lipid 4c was purified by preparative TLC. The labeled head group of lipid 4c was isolated, treated or not with JBAM, an exomannosidase without any linkage specificity, followed by treatment with HF at 0 °C to specifically cleave phosphodiesters and finally N-acetylated (NAc) using published methods (48). The generated fragments were analyzed by paper chromatography along with radiolabeled Man1–4GlcNAc–Ins standards. Their position is indicated by 0 to 4; I = Ins.
Man2. We also verified that acidic conditions per se do not affect the stability of the EtN-P groups as the incubation of cells in 5% trichloroacetic acid at 0 °C for 60 min before lipid extraction did not diminish the amount of CP lipid that could be extracted from [3H]Ins-labeled gpi8 sec18 cells (not shown). Similarly, treatment of CP2 by acetylation did not remove EtN-P from Man1 as described before (11) and this was verified during this experimental series by carrying purified CP2 through the same workup as the anchor peptides (not shown). Thus, after these preliminary tests we were confident that the enzymes used did not contain any activity transforming CP lipids into less polar compounds but we could not prevent that some substituents, nevertheless, might be lost through spontaneous hydrolysis during head group preparations.

Ethanolamine Phosphate Is Present on Man1 of the Majority of GPI Anchors in WT Cells—To detect the presence of EtN-P on Man1 we prepared anchor peptides as outlined in Fig. 3 and analyzed the resulting anchor GPI head groups by sequentially treating with acetylation, a procedure that specifically cleaves the α1–6 glycosidic bond of the anchor (Fig. 1), then JBAM and finally HF. As shown in Fig. 5, A and B, and Table III, 15% of GPI anchors of WT cells contained an HF-sensitive substituent on Man1. Using the same protocol we tested several mutants as shown in Fig. 5, B–F, and Table III, and found that the percentage of substitution on Man1 was higher in gpi7Δ and sec18-1, and that these effects were potentiated in sec18-1 gpi7Δ double mutant cells, where almost all anchors were substituted on Man1. To make sure that the low percentage of Mon1 substitution on GPI anchors of WT cells truly represented the status at the end of the metabolic labeling and were not the consequence of spontaneous or catalyzed hydrolysis during the following workup we performed the mixing experiments reported in Table IV. For this, labeled and nonlabeled cells of various genotypes were mixed before starting the workup outlined in Fig. 3. These experiments revealed neither trans-acting destabilizing factors in WT nor stabilizing factors in sec18-1 gpi7Δ cells. In addition, buffering the potential acidity of chloroform solutions with triethylamine or the inclusion of phosphodiesterase inhibitors did not influence the results. Previous studies had brought evidence for Man1-substituted complete precursor (CP) and M4 lipids but in these experiments the time of exposure of the lipids to organic solvents was significantly shorter than the time required for delipidation of proteins. We therefore continued to be concerned with the possibility of untoward hydrolysis of phosphodiester-linked side chains in organic solvents and we therefore modified the protocol of Fig. 3 by omitting the first step, i.e. the organic solvent extraction. Indeed, when labeled cells were directly boiled in SDS sample buffer, 52% of Man1 of WT anchors and 98% on sec18-1 gpi7Δ anchors were found to be substituted (Fig. 6 and Table V). Yet, by this procedure the yields of labeled anchors were 4 times lower than with the unmodified protocol. However, by either first breaking cells in Tris buffer using glass beads or preincubating cells in 0.1M sodium hydroxide before boiling in sample buffer the yields were as high as with the original procedure of Fig. 3 and yet, 72% of WT anchors were found to be substituted (Table VI). (The contribution of CP and M4 lipids to this improved yield could be excluded, as these lipids do not bind to ConA-Sepharose in the conditions used for anchor peptide preparation.) Thus, it appears that at the end of metabolic labeling, 72% of WT anchors carry a substituent on Man1 that is lost when proteins are delipidated using organic solvents. In contrast, 98% of the GPI anchors of sec18-1 gpi7Δ carry a substituent on Man1, which seems to be largely resistant to organic solvent.

HF-sensitive Substituents on Man2—We tried to investigate if GPI anchors carried any substituents on Man2 by using limiting HF digestion, a procedure that had previously been used successfully to detect an HF-sensitive substituent on Man2 of [3H]mannose-labeled CP2 of pmi40 cells (12). As can be appreciated from Fig. 7 and from the corresponding quantitation in Table VI, this method identifies a substituent on Man2 of [3H]Ins-labeled CP from gpi8-1 sec18-1 as well, but it detects only minimal amounts of substitution on Man2 of WT of sec12 protein anchors. (Mutants in SEC12 block the exit of secretory proteins out of the ER.) Small amounts of Manα2GlcNAc-Ins were also generated from M4 in this experiment (see “Discussion”). However, it appears that limiting HF does not seem to work the same way on anchor peptides as on free GPI lipids in as much as also substituents on Man1 were not detected efficiently. Indeed, with the protocol used, 15% of the anchor peptides of WT and 94% of sec18-1 gpi7Δ cells carried a

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**Fig. 3. Isolation of GPI protein anchors and generation of head groups.** After isolation, the head groups were further analyzed by chemical and enzymatic treatments as described in the text.
**TABLE II**

Degradation of CP lipids by concanavalin A-Sepharose buffer

Purified CP lipids were incubated with ConA-Sepharose or the buffer that is used normally for ConA-Sepharose affinity chromatography but without Triton X-100 and lacking some ingredients. Products were extracted, analyzed by TLC, and quantitated. All radioactivity appeared as M4/1, M4/2, or CP and the sum of these three peaks was set as 100%.

| Experiment 1 | Experiment 2 |
|--------------|--------------|
| CP M4/1 M4/2 | CP M4/1 M4/2 |
| % CP degradation | % CP degradation |
| ConA-Sepharose in buffer$^a$ | 59 2 39 41 | 42 58 |
| Buffer$^b$ | 61 3 36 39 | 50 50 |
| Buffer – Mg, – Mn | 69 2 29 31 | 10.8 |
| 50 mm Tris-HCl | 69 3 28 31 | 10.8 |
| 20 mm Tris-HCl + 0.5 mm NaCl | 18 0 82 82 | 76.5 |
| Starting CP$^c$ | 77 2 21 23 | 91 9 |

$^a$ Buffer is ConA-Sepharose buffer.

$^b$ CP lipids purified by preparative TLC is not totally stable and spontaneously starts to be contaminated by M4/1 when kept at −20 °C. In experiment 2 the CP used was freshly prepared.
Ethanolamine Phosphate Side Chains on Yeast GPI Anchors.

Fig. 5. Analysis of ethanolamine phosphate side chains on Man1 in different GPI protein anchors. X2180-1A (wt), ZY400 (mmn9), HMSF176 (sec18-1), FBY182 (gpi7Δ), and FBY49 (sec18 gpi7Δ) were labeled at 37 °C and head groups were isolated, subjected to acetylation, and then treated with JBAM. The resulting products were dephosphorylated with HF, N-acetylated, and desalted before being analyzed by paper chromatography. In panel B, JBAM treatment was omitted. Note that acetylation conditions are such that only part of the anchors are cleaved to preserve glycosidic bonds that are not α1,6. Quantitation of the data and of control experiments are reported in Table III. 0 to 4 indicate positions of standards Man0,4-GlcNAc-Ins; I = Ins.

substituent on Man1 (Table III) but only a small fraction could be detected with limiting HF. Curiously, however, in sec18-1 gpi7Δ the method detected as much substituents on Man2 as on Man1, although we know that M4, the free anchor lipid accumulating in gpi7Δ cells does not carry any substituent on Man2. As these experiments are rather extensive and the results are difficult to interpret, we did not repeat or do more experiments of this kind, but we note that with this method we detect very little substituents on Man2 in GPI anchors of WT cells. This is in agreement with the above observed lability of a substituent on Man2 of CP (Table II).

Whereas the presence of EtN-P on Man1 and Man2 of mammalian anchors is firmly established, in yeast EtN-P has formally been shown to be present only on Man1 and Man3 (11, 13, 14). The HF-sensitive substituent on Man2 was surmised to be EtN-P based on the accumulation of M4, a GPI lipid lacking a substituent on Man2, in gpi7Δ and on the homology of GPI7 with MCD4 and GPI13, which are required to add EtN-P onto Man1 and Man3, respectively (12, 15, 18, 19). However, a recent report has clearly established the presence of a GlcNAc-P residue attached to Man2 in some human GPI anchors (40). To address the question if yeast anchors carry EtN-P on Man2, sec18 gpi8-1 cells were double labeled with [3H]serine and [14C]Ins at 37 °C. The lipid extract was separated in two-dimensional TLC, the plate was radioimaged, and the spots corresponding to CP and M4 were scraped and counted in a scintillation β-counter with the windows set for separate detection of tritium and carbon 14. In M4 the [3H]:[14C] ratio was 3.0, in CP it was 4.0. As M4 has 2 and CP either 2 or 3 EtN-Ps, we would have expected that the two ratios are either the same or differ by a factor of 1.5. Yet, the labeling lasting 2 h, the specific activity of [3H]serine/[3H]PE and [14C]Ins/[14C]PI not being constant during that time, we cannot reasonably expect the ratios to differ by a factor of exactly 1.5. The higher [3H]:[14C] ratio in CP suggests that the residue on its Man2 is indeed an EtN-P.

Is CP Made in the ER and Is Gpi7p Essential for Adding the Substituent on Man2?—The foregoing and published data are compatible with the view of Gpi7p being an EtN-P transferase for Man2. Previous studies have indicated that Gpi7p is a glycoprotein located at the plasma membrane and that its N-glycans undergo extensive elongation in the Golgi. A minor core N-glycosylated form disappeared upon incubation of cells in cycloheximide (Chx) and was presumed to represent the immature ER form of Gpi7p in transit to the cell surface (12). To test the working hypothesis that ER-localized Gpi7p in transit is responsible for the transfer of the HF-sensitive substituent on Man2 of CP lipids, Gpi8p-depleted cells were labeled with [3H]Ins after a 40–70-min preincubation with Chx that ought to allow newly made Gpi7p to be transported out of the ER. This protocol leads to the complete disappearance of CP but also of the less hydrophilic GPI lipids M4/1 and M4/2 (Fig. 8, lanes 7 and 8), indicating that Chx was blocking GPI biosynthesis at an earlier stage. M4, the abnormal GPI lipid accumulating in gpi7Δ was also drastically reduced when Chx was used (Fig. 8A, lanes 7 and 8). The same phenomenon was also observed in gpi8-1 sec18-1 cells labeled at 37 °C, at which temperature all vesicular traffic is blocked. These cells made significantly more CP than gpi8-1 mutants (not shown) but Chx preincubation blocked the biosynthesis of mature GPI lipids (Fig. 8A, lanes 1 and 5). Incidentally, this result demonstrates that CP biosynthesis does not require any vesicular transport of GPI lipids out of the ER. Cycloheximide blocked GPI biosynthesis also when just added before [3H]Ins (Fig. 8A, lanes 3 and 4, and B, lane 8). Cycloheximide, however, did not induce their breakdown; as shown in Fig. 8B, M4/1, M4/2, and CP lipids, which had been accumulating over 60 min in the absence of Chx, remained entirely stable during a subsequent period of 20 min in the presence of Chx. Thus, although, as judged by Western blotting, the core-glycosylated form of Gpi7p could efficiently be accumulated by preincubation of gpi8-1 sec18-1 at 37 °C (not shown), cells were, nevertheless, unable to make mature GPI lipids in the presence of Chx. As little as 1 μg/ml Chx was sufficient to produce this effect. As shown in Fig. 9A, Chx had no effect on GPI biosynthesis when added to microsomes, raising the possibility that it was blocking the biosynthesis of glucosamine or UDP-GlcNAc. However, the addition of glucosamine (up to 100 mM) to sec18 gpi8 cells labeled with [3H]Ins in vivo did not allow cells to make M4 or CP lipids in the presence of Chx (not shown). The arrest of GPI biosynthesis caused by Chx was surprising as we previously had found that Chx stimulated the incorporation of [3H]mannose into CP and M4 lipids in pmi40, a temperature-sensitive mutant unable to make mannose at 37 °C (30, 41). We excluded a potential effect of Chx on mannose biosynthesis as the addition of mannose to the medium during [3H]Ins labeling did not allow GPI biosynthesis in the presence of Chx (not shown). One possible explanation for the discrepancy would be that the amounts of CP present in gpi8 sec18 are much higher than in pmi40. Indeed, the comparison of the ratio of [3H]CP/ [3H]M(IP)2C in [3H]mannose-labeled pmi40 with the same ratio in [3H]Ins-labeled sec18 gpi8 cells indicated that in sec18 gpi8 the CPM(IP)2C ratio is 4.5-fold higher than in pmi40. In summary, Chx seems to block an early step of GPI biosynthesis.
Ethanolamine Phosphate Side Chains on Yeast GPI Anchors

Numbers indicate the amount of Man-GlcNac-Ins as percentage of Man-GlcNac-Ins plus GlcNac-Ins after various treatments as calculated from paper chromatography profiles (1/1 + 0 in Fig. 5). As sometimes small amounts of GlcNac-Ins were present already after acetolysis/HF/NAc, the percentage of EtN-P-substituted Man1 was obtained by dividing the % of Man-GlcNac-Ins after acetolysis/JBAM/HF/NAc by the % of Man-GlcNac-Ins after acetolysis/HF/NAc. Profiles shown in Fig. 5 are in italics. The control in the third column demonstrates that all labeled head groups were completely sensitive to JBAM after HF treatment.

| Strain               | Acetolysis/JBAM/HF/NAc | Acetolysis/HF/NAc | Acetolysis/HF/NAc/JBAM | Substitution on Man1 % |
|----------------------|------------------------|-------------------|------------------------|------------------------|
| X2180–1A (wt)        | 13.8                   | 90.9              | 0                      | 15.2                   |
| ZY400 (mnn9)         | 15.3                   | 90.5              | 0                      | 16.9                   |
| FBY182 (gpi7Δ)       | 29.9                   | 91.1              | 0                      | 32.8                   |
| HMSF176 (sec18–1)    | 36.7                   | 94.9              | 0                      | 38.7                   |
| FBY49 (sec18–1 gpi7Δ)| 90.5                   | 95.8              | 0                      | 94.2                   |

**TABLE IV**

X2180–1A and sec18–1 gpi7Δ cells do not contain trans-acting factors influencing the stability of EtN-P groups during the extraction of GPI anchor head groups

X2180–1A (wt) and FBY49 (sec1–8 Δgpi7) cells were labeled for 60 min at 37 °C (labeled cells are indicated by an asterisk). After the labeling 5% of labeled cells were mixed with 5% of unlabeled cells and processed as outlined in Fig. 3. Cells were broken in chloroform/methanol, 1:1, extensively delipitated with chloroform/methanol/water, 10:10:3, then Lester solvent or alternatively with chloroform, methanol, 1.5 mM triethanolamine, pH 7.5 (10:10:3)(TEA). After solubilization of proteins in sample buffer further incubations were in some cases done in the presence of labeled at 37 °C and FBY49 cells were vents.

Analysis of protein head groups isolated avoiding organic solvents. X2180–1A and FBY49 cells were labeled at 37 °C for 1 h with [3H]Ins, cells were directly boiled in sample buffer and then further processed as outlined in Fig. 3. Head groups were subjected to acetolysis, treated or not to JBAM, products were dephosphorylated with HF, N-acetylated and desalted before being analyzed by paper chromatography. Numbers indicate the mannose and EtN-P transferases involved in GPI biosynthesis had their catalytic site on the luminal side of the ER (34, 43) these earlier data may be taken as evidence that luminally synthesized GPI lipids can flip and are in equilibrium over the two leaflets of the ER membrane. It thus appeared possible that M4 made in the ER would reach the plasma membrane through a cytosolic route and be transformed into CP by Gpi7p residing at the cell surface. To put this hypothesis to the test we asked whether the removal of Gpi7p from spheroplasts used for preparation of microsomes would

**FIG. 6.** Analysis of protein head groups isolated avoiding organic solvents. X2180–1A and FBY49 cells were labeled at 37 °C for 1 h with [3H]Ins, cells were directly boiled in sample buffer and then further processed as outlined in Fig. 3. Head groups were subjected to acetolysis, subjected or not to JBAM, products were dephosphorylated with HF, N-acetylated, and desalted prior to analysis by paper chromatography.

in vivo and we therefore could not assess the role of the ER form of Gpi7p for GPI biosynthesis in living cells.

As vesicular traffic is not required for making CP (Fig. 8A, lane 5), it seemed impossible that surface-located Gpi7p contributed to the biosynthesis of CP2 unless the GPI could reach the surface by a non-vesicular pathway. Indeed, earlier studies had shown that mature GPI lipids were accessible at the cytosolic leaflet of microsomes (42). In view of more recent data showing that the mannosyl and EtN-P transferases involved in
Experimental Procedures.

"chromatography profiles. Data in the first two rows reflect quantification of Fig. 6 and are set in italics. Proteins were isolated as described under sec18-1 gpi7 Δ cells were labeled with [3H]Ins and finally analyzed by paper chromatography. treated with JBAM, retreated with HF for 48 h, were obtained by two consecutive runs of preparative TLC. Head groups were expressed in ECM33 does not decrease, but rather increases the possibility that the transferase that adds EtN-P to normal growth at 37 °C.

Fig. 7. Presence of an HF-sensitive substituent on Man2. X2180–1A (wt) and SF226-1C (sec12-4) cells were labeled with [3H]Ins at 37 °C and anchor peptides were prepared as outlined in Fig. 3. The gpi7Δ and gpi8-1 sec18-1 cells were labeled with [3H]Ins at 37 °C to obtain radiolabeled GPI lipids M4 and CP, respectively. These lipids were obtained by two consecutive runs of preparative TLC. Head groups were treated for 12 h with HF (limiting HF treatment), desalted, treated with JBAM, retreated with HF for 48 h, N-acetylated, desalted, and finally analyzed by paper chromatography.

Effects of Overexpression of PSD1, PSD2, and ECM33 in gpi7Δ—Previous studies showed that certain mcd4 alleles render cells auxotrophic for ethanolamine if they are placed into a psd1Δ background, and other studies showed that overexpression of PSD1 can rescue the growth phenotype of a temperature-sensitive mcd4 allele (fsr2-1) (33, 37). PSD1 is the mitochondrial phosphatidylserine decarboxylase that generates the bulk of phosphatidylethanolamine, the lipid that is the immediate donor of EtN-P for Mcd4p and Gpi13p (13, 20). The above findings thus suggested that PE may be limiting for the luminal EtN-P transferase Mcd4p in certain conditions and that overexpression of PSD1 can increase luminal PE levels. Curiously, overexpression of PSD1 (or ECM33) also restored normal growth at 37 °C to gpi7Δ cells, again raising the possibility that some protein other than Gpi7p may add EtN-P to GPI anchors increases the stability of ethanolaminephosphate on Man1.

TABLE V
Omission of protein delipidation by organic solvents during the isolation of GPI anchors increases the stability of ethanolaminephosphate on Man1.

| Strains/extraction conditions | Acetolysis/JBAM/HF/NaC | Acetolysis/HF/NaC | Acetolysis/HF/NaC | Substitution on Man1 % |
|--------------------------------|------------------------|-------------------|-------------------|------------------------|
| X2180–1A (wt) boil in SDS     | 55.8 91.2 0            | 64.5 90.6 0       | 71.5               |
| sec18–1 gpi7 Δ boil in SDS   | 93.6 94.5 0            | 98.1               |
| X2180–1A (wt) break with glass beads in Tris buffer, then boil in SDS | 65.4 91.5 0 | 71.5 | |
| X2180–1A, treated with 0.1 M NaOH for 5 min, boil in SDS | 64.5 90.6 0 | 71.2 | |

Numbers indicate the Man-GlcNAc-Ins as percentage of Man-GlcNAc-Ins plus GlcNAc-Ins after various treatments as calculated from paper chromatography. Figures 7 and 8 complement these data. The results in Table V show that omission of organic solvent treatment during the isolation of GPI anchors increases the stability of ethanolaminephosphate on Man1.

We tried to get independent evidence for transfer of GPI lipids between microsomes during the in vitro GPI biosynthesis assay by mixing microsomes from gpi mutants blocked at various stages of the biosynthetic pathway. As shown in Fig. 9B, there was no biochemical complementation of gpi7Δ microsomes by microsomes from gpi1 or gpi10 cells in that these mixtures did not produce any CP and this was true even though concentrated cytosol (a source of possible lipid transfer proteins) had been added to the standard assays. In summary these studies indicate that the microsomal GPI biosynthesis assay generates CP in the absence of any vesicular fusion or transfer of GPI intermediates from one vesicle to the other suggesting the possibility that the transferase that adds a substitute to Man2 in microsomes is not Gpi7p.

The absence of Cspheroplasts (Fig. 9C, lanes 2 and 4). Although the inhibition of GPI biosynthesis by Chx is not clearly understood, we tried to ask if preincubating living cells with Chx would affect a subsequent microsomal GPI biosynthesis assay. Experiments to address this question are shown in Fig. 9C, and are quantitated in Table VII. It clearly appears that microsomes prepared from cells preincubated with Chx incorporate less [3H]GlcNAc into CP and the same applies to M4 lipids. However, even the combination of Chx preincubation and the use of Zymolyase did not result in a complete suppression of GPI biosynthesis (lane 5). Thus, whereas Zymolyase and Chx are efficient in eliminating the high molecular weight and core-glycosylated form of Gpi7p (data not shown), their combined action did not completely abrogate the biosynthesis of CP.

To test for independent evidence for transfer of GPI lipids between microsomes during the in vitro GPI biosynthesis assay by mixing microsomes from gpi mutants blocked at various stages of the biosynthetic pathway. As shown in Fig. 9B, there was no biochemical complementation of gpi7Δ microsomes by microsomes from gpi1 or gpi10 cells in that these mixtures did not produce any CP and this was true even though concentrated cytosol (a source of possible lipid transfer proteins) had been added to the standard assays. In summary these studies indicate that the microsomal GPI biosynthesis assay generates CP in the absence of any vesicular fusion or transfer of GPI intermediates from one vesicle to the other suggesting the possibility that the transferase that adds a substitute to Man2 in microsomes is not Gpi7p.

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Effects of Overexpression of PSD1, PSD2, and ECM33 in gpi7Δ—Previous studies showed that certain mcd4 alleles render cells auxotrophic for ethanolamine if they are placed into a psd1Δ background, and other studies showed that overexpression of PSD1 can rescue the growth phenotype of a temperature-sensitive mcd4 allele (fsr2-1) (33, 37). PSD1 is the mitochondrial phosphatidylserine decarboxylase that generates the bulk of phosphatidylethanolamine, the lipid that is the immediate donor of EtN-P for Mcd4p and Gpi13p (13, 20). The above findings thus suggested that PE may be limiting for the luminal EtN-P transferase Mcd4p in certain conditions and that overexpression of PSD1 can increase luminal PE levels. Curiously, overexpression of PSD1 (or ECM33) also restored normal growth at 37 °C to gpi7Δ cells, again raising the possibility that some protein other than Gpi7p may add EtN-P to GPI lipids if the PE levels are high enough (33). The experiment shown in Fig. 10A indicates that the overexpression of PSD1 or ECM33 does not decrease, but rather increases the accumulation of M4 in gpi7Δ cells and that it does not lead to the accumulation of CP lipids in a thermosensitive gpi7 gpi8 double mutant, a mutant, which accumulates CP when complemented with Gpi7 (12). Similarly, the overexpression of PSD2, a phosphatidylserine decarboxylase of the secretary apparatus, does not abolish the accumulation of M4 when overexpressed in gpi7Δ that simultaneously overexpress Mcd4 or ECM33 (not shown). Thus, overexpression of PSD1 or ECM33 seem to improve the growth of gpi7Δ cells not by allowing for CP biosynthesis but rather by increasing M4 biosynthesis sug-
gesting that lumenal PE levels may be limiting for Mcd4p or Gpi13p, at least in gpi7/H9004 cells. However, it cannot be completely excluded that a small amount of CP, not detectable in gpi7 gpi8, is generated and used to make critical amounts of certain cell wall proteins that require complete anchors for function. Candidate genes for adding EtN-P in the absence of Gpi7p would be Mcd4p and Gpi13p. As shown in Fig. 10B, overexpression of Gpi13p can slightly improve the growth of gpi7. Overexpression of MCD4 in gpi7Δ cells may be a physiological phenomenon as deletion of GPI7 ought to induce MCD4; indeed, we found that deletion of GPI7 induces a strong unfolded protein response (UPR) (not shown), a fact that is in agreement with the previously reported dependence of gpi7Δ on Ire1p (44), and the UPR has been reported to very strongly induce MCD4 but not GPI13 (45). Depletion of Mcd4p or Gpi13p also induced a strong UPR (not shown). Our results

Table VI

Quantitation of data of Fig. 7

FBY49 (sec18–1 gpi7Δ) cells were labeled and processed along with other cells shown in Fig. 7. Boxheads indicate the various species of Manx-GlcNAc-Ins containing from 0 to 4 mannoses. Numbers indicate the fraction of Manx-GlcNAc-Ins as a percentage of all peaks. Values obtained from the profile of Fig. 7 are in italics.

| Strain                        | x = 4 | x = 3 | x = 2 | x = 1 | x = 0 |
|-------------------------------|-------|-------|-------|-------|-------|
| X2180–1A (wt)                 | 0     | 38.8  | 1.9   | 0     | 59.3  |
| sec12–4                       | 0     | 46.3  | 3.5   | 9.2   | 41.0  |
| sec18–1 gpi7Δ                 | 2.8   | 41.3  | 12.2  | 12.4  | 31.3  |
| M4 of gpi7Δ                   | 2.3   | 22.2  | 5.3   | 11.1  | 59.1  |
| CP of gpi8–1 sec18–1          | 2.4   | 33.8  | 27.7  | 16.0  | 20.1  |

Fig. 8. Preincubation of gpi8–1 with cycloheximide abolishes biosynthesis of CP and M4. A. gpi8 sec18 cells, FBY166 (gpi8Δ) cells depleted of Gpi8p by growing on glucose for 24 h at 24 °C and gpi7Δ cells were preincubated and labeled with [3H]Ins at 24 or 37 °C in the presence or absence of 100 μg/ml cycloheximide as indicated. FBY166 and cells in lane 4 were preincubated for 70 min, all other cells for 40 min. Lipids were run on TLC in solvent 1. Free Ins is present because of incomplete desalting. B, gpi8 sec18 were incubated for the indicated times in the presence of 20 μCi of [3H]Ins and lipids were analyzed as in A. As indicated, sample 6 received Chx after 60 min of labeling, sample 7 was preincubated to accumulate Gpi7p in the ER, sample 8 received Chx 5 min before [3H]Ins. Incorporation of label was monitored by measuring the radioactivity in the desalted lipid extract.
cannot completely exclude that small amounts of EtN-P may be added to Man2 by Gpi13p or Mcd4p but on the whole it appears that Mcd4p, Gpi7p, and Gpi13p exert rather different functions. We incidentally also tested if overexpression of Gpi7p would rescue either mcd4Δ or gpi13Δ mutants. In mcd4Δ or gpi13Δ, the endogenous Gpi7p may fail to add EtN-P onto Man1 or Man3 not because of its inappropriate specificity but because of its inappropriate location outside the ER. We had noted before that the overexpression of GPI7 results in the accumulation of Gpi7p in a core-glycosylated form (51). Yet, as shown in Fig. 10B, overexpression of GPI7 did not improve the growth of Mcd4p- or Gpi13p-depleted cells.

Identification of Functional Domains in Gpi7p—Our previous study (12) on Gpi7p had revealed that the protein, apart from EtN-P transfer, was required for a second enzymatic activity, the remodeling (exchange) of the lipid moiety of GPI anchors in the late compartments of the secretory pathway. The structure of Gpi7p contains a 400-amino acid globular ectocytolic domain followed by a 430-amino acid long hydrophobic domain containing 8–11 membrane spanning regions (Fig. 11A). The Gpi7p constructs shown in Fig. 11A were introduced into gpi7Δ cells to test their capacity to abolish the abnormal accumulation of the GPI lipid M4 and to restore GPI anchor remodeling. Western blotting with anti-Gpi7p antibody demonstrated that all constructs were expressed and that the products of pBF118 and pBF119 were only core glycosylated, suggesting that they are efficiently retained in the ER, whereas those of pBF121 and pBF122 were of high molecular weight indicating that the KKKX motif added to the C terminus of Gpi7p was luminal or otherwise non-functional (Fig. 11B). M4 accumulation was suppressed by pBF43, pBF120, pBF121, and pBF122, whereas all other constructs, even when overexpressed, were inefficient in this respect (not shown). Similarly, only WT Gpi7p forms could restore remodeling of GPI proteins and calcifluor resistance (not shown). In summary, we could not dissect a protein into functional domains simply by making the large deletions indicated in Fig. 11A.

**DISCUSSION**

The finding that the addition of EtN-P to Man1 of yeast GPI lipids is an obligatory step of GPI biosynthesis in yeast led us to reinvestigate the structure of the yeast GPI anchor and led us to the conclusion that indeed, most GPI anchors contain an HF-sensitive substituent on Man1. The study also led us to realize some intricacies that render the study of EtN-P substituents tricky and may explain the substoichiometric amounts of EtN-P substituents found in GPI anchors of other organisms. Indeed, the substituent on Man1 is relatively labile during lengthy delipidation of GPI proteins in organic solvents, but it somehow gets resistant to organic solvents in gpi7Δ sec18 mutants. The effect of the gpi7Δ sec18 double mutation is discernible even if organic solvents were avoided, in which case 98% of anchors had a substitution on Man1 in gpi7Δ sec18 but only 72% in WT (Table V). There is no obvious explanation for this finding but several hypotheses can be formulated: 1) if there is no substitution on Man2 the stability of the EtN-P on Man1 may increase because of altered electrostatic interactions. Alternatively, a spontaneous transesterification reaction that translocates the EtN-P from the C atom it was attached to by Mcd4p to another C atom may be enhanced or prevented by the presence of a substituent on Man2. 2) Deletion of GPI7 induces the cell wall integrity pathway (46), and the UPR. These responses may induce enzymes that change the configuration of the substituent on Man1. 3) The small amounts of Gpi7p present in the ER may not, or not only be important for the addition of EtN-P on Man2 but may also hydrolyze or transesterify the EtN-P residue on Man1. Absence of Gpi7p thus could stabilize the EtN-P on Man1. It must be said that it is unclear if the substituent is primarily stable and loses stability in the context of WT cells, or it is primarily unstable and gains stability in the context of gpi7Δ sec18 cells. It is also surprising that the substituent is only partially stabilized in gpi7Δ single mutants indicating that the environment inside and outside the ER influences the substituent on Man1 differently. Clearly, to resolve this puzzle, it will be necessary to compare the site of attachment of the Man1 substituent on anchors of WT and gpi7Δ sec18 mutants. (The short exposure to organic solvents required for lipid extraction leaves the Man1 substituents of CP intact but it could well be that part of CP initially present after labeling looses its substituent and is transformed into M4/2 (Fig. 8A) during extraction.)

The substituent on Man2 most likely is an EtN-P, but it is at present not clear if this substituent is stably associated with...
**Fig. 10. Depletion of Gpi13p and Mcd4p in cells overexpressing Gpi7p.** A, FBY115 (gpi7Δ, lanes 1–3), YAT2626 (gpi7Δ, lanes 4–6), and FBY122 (gpi13Δ, lanes 7–12) were transformed with multicopy vectors containing PSD1 (P), ECM33 (E), or nothing (Φ) and were labeled at the indicated temperature with [3H]Ins. Lipids were extracted and analyzed by TLC/fluorography. The relative percentage of M4 lipid as compared with the total of radioactivity per lane is given at the bottom of each lane. B, 10-fold dilutions of FBY1104 (mcd4Δ::HIS3 GAL1::MCD4), FBY1102 (gpi13Δ::HIS3-GAL1::GPI13), and FBY15 (gpi7Δ) harboring MCD4, GPI13, and GPI7 behind their own promoter on a URA3-based 2μ multicopy vector or harboring empty vector (Φ) were spotted on plates containing galactose or glucose and calcofluor white as indicated on the top of each column and were incubated at 30 °C for 3 days.

**TABLE VII**

| Preincubation of cells with Chx | Experiment 1 | Experiment 2 | Experiment 3 |
|--------------------------------|--------------|--------------|--------------|
| | CP | M4/1 + M4/2 | CP | M4/1 + M4/2 | CP | M4/1 + M4/2 |
| Quantazyme No Chx | 20.5 | 6.4 | 17.1 | 7.1 | 26.3 | 6.5 |
| Yes Chx | 7.8 | 4.9 | 15.2 | 5.4 |
| Zymolyase No Chx | 10.7 | 4.5 | 7.1 | 3.6 | 8.5 | 3.6 |
| Yes Chx | 5.1 | 3.3 | 4.1 | 2.1 |

Influence of Zymolyase and cycloheximide (Chx) preincubation on biosynthesis of GPI lipids in vitro

Radioscanning of TLC plates allowed to quantitate the relative amount of CP and M4 lipids as % of total radioactivity in a given lane for the experiment shown in Fig. 9, A and C (here experiments 1 and 3, respectively) and a further identical experiment. Values obtained by scanning the fluorograms of Fig. 9 are in italics.
or WT cells (Table VI). 3) CP lipid was made but more substituents on Man2 than anchors from sec12 Man4-GlcN-Ins core with a single EtN-P on either Man1 (80%) accumulating in Gpi13p-depleted cells was shown to contain a,

Microsomes, derived from cells that had lost Gpi7p through loss of a substituent on Man2 in the ER, this also may second-

under physiological conditions the substituent is stable but it may not be the active component of the transerase as it does not have a phosphodiesterase motif and, based on studies in mammalian cells, is believed to be required for stabilization of the EtN-P transferases (18). We suspected that Mcd4p and/or Gpi13p might serve this function in the absence of Gpi7p. However, overexpression of these genes improved the growth of gpi7 mutants on calciofluor white only very little. Thus, whatever the identity of the EtN-P transerase for Man2, its activity remains dependent on Gpi7p. The role of Gpi13p, Gpi7p, and Mcd4p in the transfer of EtN-P to GPI anchors will have to be further studied by in vitro activity tests with purified enzymes and substrates to unambiguously establish their exact function in this process.

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**REFERENCES**

1. McConville, M. J., and Ferguson, M. A. (1993) Biochem. J. 294, 305–324
2. Takeda, J., and Kinoshita, T. (1995) Trends Biochem. Sci. 20, 367–371
3. McConville, M. J., Collidge, T. A., Ferguson, M. A., and Schneider, P. (1993) J. Biol. Chem. 268, 15595–15604
4. Homans, S. W., Ferguson, M. A., Drew, R. A., Redemann, T. W., Anand, R., and Williams, A. F. (1988) Nature 339, 269–272
5. Roberts, W. L., Santikarn, S., Reinhold, V. N., and Rosenberry, T. L. (1988) J. Biol. Chem. 263, 18776–18784
6. Puoti, A., Despods, C., Fankhauser, C., and Conzelmann, A. (1991) J. Biol. Chem. 266, 21051–21059
7. Hirase, S., Prince, G. M., Selevyer, D., Ravi, L., Rosenberry, T. L., Ueda, E., and Medof, M. E. (1992) J. Biol. Chem. 267, 16968–16974
8. Kamitani, T., Menon, A. K., Hallay, Y., Warren, C. D., and Yeh, E. T. (1992) J. Biol. Chem. 267, 24611–24619
9. Puoti, A., and Conzelmann, A. (1993) J. Biol. Chem. 268, 7215–7224
10. Ueda, E., Selevyer, D., Prince, G. M., Rosenberry, T. L., Hirase, S., and Medof, M. E. (1993) J. Biol. Chem. 268, 9998–10002
11. Canivecz-Gansel, E., Imhof, I., Begu, P., Conzelmann, A., and Benachour, A. (1998) Glycobiology 8, 761–770
12. Benachour, A., Sigos, G., Flury, I., Reggiori, F., Canivecz-Gansel, E., Vionnet, C., Conzelmann, A., and Bengherzal, M. (1999) J. Biol. Chem. 274, 15251–15261
13. Imhof, I., Canivecz-Gansel, E., Meyer, U., and Conzelmann, A. (2000) Glycobiology 10, 1271–1275
14. Fankhauser, C., Homans, S. W., Thomas-Oates, J. E., McConville, M. J., Despods, C., Conzelmann, A., and Ferguson, M. A. (1993) J. Biol. Chem. 268, 26365–26374
15. Gaynor, E. C., Mondesert, G., Grimm, S. M., Reed, S. I., Orlean, P., and Emr, S. D. (1999) Mol. Biol. Cell 10, 627–648
16. Hong, Y., Maeda, Y., Watanabe, R., Oishi, K., Mishkind, M., Riezman, H., and Kinoshita, T. (1999) J. Biol. Chem. 274, 35099–35106
17. Hong, Y., Maeda, Y., Watanabe, R., Inoue, N., Oishi, K., and Kinoshita, T. (2000) J. Biol. Chem. 275, 20911–20919
18. Taron, C. H., Wiedman, J. M., Grimme, S. J., and Orlean, P. (2000) Mol. Biol. Cell 11, 1611–1630
19. Flury, I., Benachour, A., and Conzelmann, A. (2000) J. Biol. Chem. 275, 24456–24465
20. Menon, A. K., and Stevens, V. L. (1992) J. Biol. Chem. 267, 15277–15280
21. Menon, A. K., Eppinger, M., Mayor, S., and Schwarz, R. T. (1993) EMBO J. 12, 1907–1914
22. Sugiyama, E., DeGasperi, R., Urayake, M., Chang, H. M., Thomas, L. J., Hyman, R., Warren, C. D., and Yeh, E. T. (1991) J. Biol. Chem. 266, 12119–12122
23. Imai, N., Kinoshita, T., Orii, T., and Takeda, J. (1993) J. Biol. Chem. 268, 6882–6885
24. Toh-e, A., and Oguchi, T. (1998) Genes Genet. Syst. 73, 365–375
25. Sherman, F. (2002) Methods Enzymol. 350, 3–41
26. Guillot, I., Pfefferli, M., and Conzelmann, A. (2000) Methods Enzymol. 312, 306–315
27. Hanson, B. A., and Lester, R. L. (1980) J. Lipid Res. 21, 509–515
28. Roberts, W. L., Santikarn, S., Reinhold, V. N., and Rosenberry, T. L. (1992) J. Biol. Chem. 263, 18766–18775

**Fig. 11.** Dissection of Gpi7p into partially functional domains. A, the indicated constructs were constructed and expressed in gpi7Δ mutants (FBJ182). A plus sign (∗) indicates the position of the conserved phosphodiesterase/nucleotidepyrophosphatase motif, whereas a asterisk (∗) indicates the two motifs of the globular domain that are generally conserved in the MCD4/GPI13/GPI13 family (12, 47). The conserved FBP-B 2878 domain extends from position 77 to 276. Deletions are in black, the hydrophobic C terminus is hatched. B, cells expressing the various constructs were extracted with sample buffer containing 20 mM EDTA and probed by Western blotting using affinity purified anti-Gpi7p (12).
30. Sipos, G., Pusti, A., and Conzelmann, A. (1994) *EMBO J.* **13,** 2789–2796
31. Davitz, M. A., Hom, J., and Schenkman, S. (1989) *J. Biol. Chem.* **264,** 13760–13764
32. Menzel, R., Vogel, F., Kargel, E., and Schunck, W. H. (1997) *Yeast* **13,** 1211–1229
33. Toh-e, A., and Oguchi, T. (2002) *Genes Genet. Syst.* **77,** 309–322
34. Takahashi, M., Inoue, N., Ohashi, K., Maeda, Y., Nakamura, N., Endo, Y., Fujita, T., Takeda, J., and Kinoshita, T. (1996) *EMBO J.* **15,** 4254–4261
35. Sutterlin, C., Escrivano, M. V., Gerold, P., Maeda, Y., Mazon, M. J., Kinoshita, T., Schwarz, R. T., and Riezman, H. (1998) *Biochem. J.* **332,** 155–159
36. Sutterlin, C., Horvath, A., Gerold, P., Schwarz, R. T., Wang, Y., Dreyfass, M., and Riezman, H. (1997) *EMBO J.* **16,** 6374–6383
37. Storey, M. K., Wu, W. I., and Voelker, D. R. (2001) *Biochim. Biophys. Acta* **1532,** 234–247
38. Packeiser, A. N., Urakov, V. N., Poljakova, Y. A., Shimanova, N. I., Shcherbukhin, V. D., Smirnov, V. N., and Ter-Avanesyan, M. D. (1999) *Yeast* **15,** 1485–1501
39. Grimme, S. J., Westfall, B. A., Wiedman, J. M., Taron, C. H., and Orlean, P. (2001) *J. Biol. Chem.* **276,** 27731–27739
40. Fukushima, K., Ikehara, Y., Kanai, M., Kochibe, N., Kuroki, M., and Yamashita, K. (2003) *J. Biol. Chem.* **278,** 36296–36303
41. Payton, M. A., Rheinheimer, M., Klig, L. S., DeTiani, M., and Bowden, E. (1991) *J. Bacteriol.* **173,** 2066–2070
42. Vidugiriene, J., and Menon, A. K. (1994) *J. Cell Biol.* **127,** 333–341
43. Maeda, Y., Watanabe, R., Harris, C. L., Hong, Y., Ohishi, K., Kinoshita, K., and Kinoshita, T. (2001) *EMBO J.* **20,** 250–261
44. Ng, D. T., Spear, K. D., and Walter, P. (2000) *J. Cell Biol.* **150,** 77–88
45. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000) *Cell* **101,** 249–258
46. Toh-e, A., and Oguchi, T. (2001) *Genes Genet. Syst.* **76,** 383–410
47. Richard, M., De Groot, P., Courtin, O., Pouain, D., Klis, F., and Gaillardin, C. (2002) *Microbiology* **148,** 2125–2133
48. Ferguson, M. A., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1988) *Science* **239,** 753–759
49. Benghezal, M., Benachour, A., Rusconi, S., Aebi, M., and Conzelmann, A. (1996) *EMBO J.* **15,** 6575–6583
50. Meyer, U., Benghezal, M., Imhof, I., and Conzelmann, A. (2000) *Biochemistry* **39,** 3461–3471
51. Fraering, P. (2001) *Functional Analysis of the Yeast Saccharomyces cerevisiae Gpi8 Protein and Characterization of the Purified Gpi Transamidase Complex.* Ph.D. thesis, University of Fribourg, Fribourg, Switzerland
Glycosylphosphatidylinositol (GPI) Proteins of Saccharomyces cerevisiae Contain Ethanolamine Phosphate Groups on the α1,4-linked Mannose of the GPI Anchor
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