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Deletion of GPI7, a Yeast Gene Required for Addition of a Side Chain to the Glycosylphosphatidylinositol (GPI) Core Structure, Affects GPI Protein Transport, Remodeling, and Cell Wall Integrity*

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Gpi7 was isolated by screening for mutants defective in the surface expression of glycosylphosphatidylinositol (GPI) proteins. Gpi7 mutants are deficient in YJL062w, herein named GPI7. GPI7 is not essential, but its deletion renders cells hypersensitive to Calcofluor White, indicating cell wall fragility. Several aspects of GPI biosynthesis are disturbed in Δgpi7. The extent of anchor remodeling, i.e. replacement of the primary lipid moiety of GPI anchors by ceramide, is significantly reduced, and the transport of GPI proteins to the Golgi is delayed. Gpi7p is a highly glycosylated integral membrane protein with 9–11 predicted transmembrane domains in the C-terminal part and a large, hydrophilic N-terminal ectodomain. The bulk of Gpi7p is located at the plasma membrane, but a small amount is found in the endoplasmic reticulum. GPI7 has homologues in Saccharomyces cerevisiae, Caenorhabditis elegans, and man, but the precise biochemical function of this protein family is unknown. Based on the analysis of M4, an abnormal GPI lipid accumulating in gpi7, we propose that Gpi7p adds a side chain onto the GPI core structure. Indeed, when compared with complete GPI lipids, M4 lacks a previously unrecognized phosphodiester-linked side chain, possibly an ethanolamine phosphate. Gpi7p contains significant homology with phosphodies terases suggesting that Gpi7p itself is the transferase adding a side chain to the α1,6-linked mannose of the GPI core structure.

Glycosylphosphatidylinositol (GPI)-anchored proteins represent a subclass of surface proteins found in virtually all eukaryotic organisms (1). The genome of Saccharomyces cerevisiae contains more than 70 open reading frames (ORFs) encoding for proteins that, as judged from the deduced primary sequence, can be predicted to be modified by the attachment of a GPI anchor (2, 3). In about 25 of them, the presence of an anchor has been confirmed biochemically. A majority of them lose part of the anchor and become covalently attached to the β1,6-glucans of the cell wall (4–6). A minority of GPI proteins retain the GPI anchor in an intact form and stay at the plasma membrane (PM).

For the biosynthesis of GPI anchors, phosphatidylinositol (PI) is modified by the stepwise addition of sugars and ethanolamine phosphate (EtN-P), thus forming a complete precursor lipid (CP) which subsequently is transferred en bloc by a transamidase onto newly synthesized proteins in the ER (7, 8). The identification of genes involved in the biosynthesis of the CP and its subsequent attachment to proteins has been possible through the complementation of mammalian and yeast gpi mutants, i.e. mutants being deficient in GPI anchoring of membrane structures (7, 9–20). In our laboratory, a series of recessive gpi mutants (gpi4 to gpi10) has been obtained by screening for yeast mutants that are unable to display the GPI-anchored α-agglutinin (Sag1p) at the outer surface of the cell wall, although the synthesis and secretion of soluble proteins is normal (21, 22).

Here we report on the characterization of gpi7. Four independent gpi7 mutants accumulated M4, an abnormal GPI intermediate that is less hydrophilic than CP2, the precursor accumulating when the transfer of GPIs to proteins is interrupted (18, 19, 21, 23). Our preliminary characterization of M4 had shown that deacylation by NH3 followed by HF treatment, used to hydrolyze selectively the phosphodiester bonds (Fig. 1), yielded the same Man4-GlcN-inositol fragment as CP2, and we speculated that gpi7 mutants may be unable to add the EtN-P onto Man3 (Fig. 1) (21). Here we show that this speculation was wrong, that CP2 differs from M4 with regard to a previously unrecognized side chain attached to Man2 (Fig. 1), and that GPI7 is required for the attachment of this side chain.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials—S. cerevisiae strains were FY611 [MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi7-1], FY615 [MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi7-1], W303-1B (MATa ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15), erosphospholipid 1; PI, phosphatidylinositol; PM, plasma membrane; ts, thermosensitive; wt, wild type; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; nt, nucleotide; PCR, polymerase chain reaction; kb, kilobase pair(s); HPLC, high pressure liquid chromatography.
X2150-1 (MATa lys), FYB118 (MATa ade2-1 ura3-1 leu2-3,112 trpl-1 his3-11,15 gpi8-1 gpi7-1), FYB182 (MATa ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7-1::KanMX4), HMSF176 (MATa sec18-1), FYB49 (MATa sec18-1 gpi7::KanMX4), C4 (MATa ura3-52 leu2-3,112 pmi40), HMSF331 (MATa sec36-3), LB1234-3B (MATa mnn9), and YNS7-A (MATa ura3 his-3 ncl1 och1::LEU2). Diploid strains were FYB118 (MATa ade2-1 ade2-1 ura3-1 ura3-1 leu2-3,112 leu2-3,112 TRP1 trpl-1 his3-11,15 lys/lys), FYB40 (MATa ade2-1 ade2-1 ura3-1 ura3-1 leu2-3,112 leu2-3,112 TRP1 trpl-1 his3-11,15 lys/lys), YNS4 (MATa ade2-1 ade2-1 ura3-1 ura3-1 ura3-1 leu2-3,112 leu2-3,112 TRP1 trpl-1 his3-11,15 lys/lys). GPI Anchor Side Chains

**Fig. 1. Presumed structure of the complete yeast precursor glycolipid GPi7.** Relevant cleavage procedures are indicated: Man1, Man2, Man3, and Man4 designate the α,1,4-linked, α,1,6-linked, and α,1,2-linked mannoses (Man). X indicates an HF-sensitive group that is not yet defined chemically. A, alkyl; P, phosphate.

**Membrane Association, Protease Sensitivity, and Cellular Localization of Gpi7p—**The nature of the association of Gpi7p with the membrane was assessed using a 100 µg of recombinant protein. For mapping of the recombinant protein were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, and anti-serum was affinity purified as described (29).

**Materials**

The nature of the association of Gpi7p with the membrane was assessed using a 100 µg of recombinant protein. For mapping of the recombinant protein were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, and anti-serum was affinity purified as described (29).
nentially growing cells were radiolabeled at 37 °C with [3H]Ins (2 μCi/A₅₀₀), and desalted lipid extracts were analyzed by TLC (solvent 2) and fluorography. The same amount of radioactivity was spotted in each lane. M(IP)₂C₆, inositol phosphomannosylinositol phosphophceramide. The upper part of the fluorogram was scanned at higher sensitivity to bring into view the faint bands of M₀. Lane 10 contains [3H]Ins. Samples still contain residual amounts of free [3H]Ins after extraction into butanol. The band migrating between CP₂ and M₄ in paper chromatography as described above. Samples were then dried in the Speed-Vac and treated with JBAM prior to complete HF dephosphorylation (60 h, 0 °C). Samples were neutralized 8-ml Sephadex G-10 (Amersham Pharmacia Biotech) column. Samples were prepared from labeled proteins as described (23). Anchor peptides were prepared exactly as described (23).

RESULTS

Cloning of GPI7—As reported before (19) and shown in Fig. 2, wild type (wt) cells do not contain polar GPIs (lane 1), gpi8-1 accumulates CP₂ as the most polar GPI lipid (lane 8), and gpi7-1 and the gpi7-1/gpi8-1 double mutant accumulate M₄ (lanes 4 and 6), thus demonstrating that gpi7-1 is epistatic to gpi8-1 and suggesting that, during GPI biosynthesis, GPI7 may act before Gpi8p. Although the original gpi7 mutants and the unrelated gpi8-1 mutant were not significantly temperature-sensitive (ts) for growth, the growth of the gpi7-1/gpi8-1 double mutant was strongly temperature-dependent. Transfection of a genomic library into this double mutant allowed the isolation of clones containing complementing plasmids (19).

These clones were labeled with myo-[3H]inositol ([3H]Ins) at 37 °C, and the lipids were extracted and analyzed by TLC. Upon transfection some gpi7-1/gpi8-1 indeed had regained the ability to make CP₂ (Fig. 2, lanes 6 and 7) and showed the same lipid profile as gpi8-1 (Fig. 2, lane 8). All these clones harbored plasmids containing YJL062w as the only complete ORF. Transfection of a multicopy vector containing YJL062w under its own promoter (pBF41, Fig. 3C) into gpi7-1 almost completely cured the accumulation of M₄ (Fig. 2, lane 5). As expected, the accumulation of CP₂ by gpi8-1 was not abolished by the overexpression of YJL062w (Fig. 2, lanes 8 and 9). YJL062w predicts an 830-amino acid membrane protein with an N-terminal signal sequence for insertion into the ER, 5 potential N-glycosylation sites for, and about 9–11 putative transmembrane domains (Fig. 3, A and B). YJL062w was deleted and replaced by the selectable marker KanMX4. On rich medium the deletants grew about as rapidly as wt cells at all temperatures. Thus, YJL062w is not an essential gene. We were unable to sporulate ΔYJL062/ΔYJL062 diploids indicating that YJL062 is required for sporulation. However, ΔYJL062/ΔYJL062 heterozygotes sporulated readily, and ΔYJL062 spores germinated normally. In accordance with previous results on gpi7 mutants (21), growth of ΔYJL062 (Δgpi7, see below) on plates at 37 °C was severely inhibited by 0.5 mg/ml Calcofluor White. ΔYJL062 accumulated M₄ at even higher levels than gpi7-1, and this accumulation was almost completely suppressed by the transfection of pBF41 (Fig. 2, lanes 2 and 3). Residual accumulation of M₄ may be due to

1. Flury, unpublished observations.
some cells that lost the complementing plasmid. Transfection of YJL062w under its own promoter on a single copy vector (plasmid pBF43) was sufficient to suppress the accumulation of M4 in a homozygous ΔYJL062/ΔYJL062 diploid (Fig. 2, lanes 11 and 12). As can be seen in Fig. 2, gpi7-1, ΔYJL062, ΔYJL062/ΔYJL062, and gpi8-1 mutants also show minor amounts of the GlcNa1,6(acyl→)Ins-P-DAG GPI intermediate M0, the accumulation of which is believed to reflect a build up of GPI intermediates throughout the biosynthetic pathway (Fig. 2, lanes 2, 4, 6, 8, and 11). (It should be noted that some intermediates of intermediate size are obscured on TLC by PI and inositol phosphoceramide (41).) As expected, expression of YJL062w abolishes the accumulation of M0 in gpi7-1 and ΔYJL062 (Fig. 2, lanes 3, 5, and 12) but not in gpi7-1/gpi8-1 nor gpi8-1 (lanes 7 and 9), since in the latter the GPI biosynthesis remains blocked. To evaluate if the mutation in gpi7-1 is genetically linked to YJL062w, YJL062w was disrupted in a heterozygous gpi7-1/GPI7 diploid. Correct replacement of one YJL062w locus was verified by PCR in two independent genetic-resistant transformants. The verified deletants were sporulated, and a total of 26 complete tetrads was labeled with [3H]Ins to analyze the accumulation of M4. In all 26 tetrads only two of the four segregants showed accumulation of M4, whereas the other two showed the lipid profile of wt cells. Geneticin resistance also segregated 2:2 and cosegregated with M4 accumulation in all cases. This demonstrates that the mutation of gpi7-1 is tightly linked to YJL062w which we henceforth call GPI7. Since a construct containing only 348 nucleotides 5' of the initiation codon of GPI7 still retained significant complementing activity, we also can dismiss the possibility that the complementing activity of pBF41 is due to one of the two small ORFs located on the opposite strand in the 5' upstream region of GPI7 and starting at −409 and −503 with regard to the start codon of GPI7.

Characterization of the GPI Intermediate M4—We found that M4, contrary to our initial expectation, contained an HF-sensitive group on Man3 (Fig. 1). Indeed, treatment of the lipid extracts of gpi7-1 with jack bean α-mannosidase (JBAM, an exomannosidase) shifted M4 to a slightly less hydrophilic position on TLC (Fig. 4A, lanes 1 and 2) but not to the position of M0. It seemed conceivable that JBAM did not remove more than one Man from M4 because it was sterically hindered by the detergent micelle in which M4 was embedded. To circumvent this problem, M4 was purified by preparative TLC, and its hydrophilic head group was liberated by GPI-PLD, O-deacylated by NH3, and then subjected to several treatments as indicated at the top of Fig. 5, A–D. The N-acetylated fragment comigrated with the Manα-GlcNaC-Ins standard (Fig. 5A). When treated with JBAM before HF, the resulting N-acetylated fragment comigrated with the Manα-GlcNaC-Ins standard, clearly indicating the presence of a blocking group on Man3 (Fig. 5B). The blocking group on Man3 was HF-sensitive, since JBAM done after HF produced a fragment comigrating with GlcNaC-Ins (Fig. 5C). Aspergillus satoi α-mannosidase (ASAM), a linkage-specific α,1,2-exomannosidase, when used after HF treatment, produced Manα-GlcNaC-Ins (Fig. 5D). The migration of the fragments shown in Fig. 5, A and B, was much slower when N-acetylation was omitted (not shown). This partial characterization of M4 is consistent with the presence of a classical Manα1,2/EtN-P→Manα1,2Manα1,6Manα1,4-GlcNa1,6Ins core structure. Having recently discovered an additional EtN-P on Man1 of CP2 (22), we considered the possibility that M4 may be lacking EtN-P on Man1. We thus proceeded to compare the non-dephosphorylated head groups of M4 and CP2 by Dionex HPLC using a system in which the presence of negatively charged phosphodiesteric bonds greatly retards the elution of oligosaccharides (42). The non-dephosphorylated head groups of M4 and CP2 eluted as sharp peaks at fractions 22 and 31, respectively (not shown). This wide separation suggested that the head group of M4 contains less negative charge than the one of CP2. To assay directly for a side chain on Man1 of M4, the head group of [3H]Ins-labeled M4 was first cleaved by acetylation, a procedure which, under mild conditions, selectively cleaves α1,6-glycosidic bonds (Fig. 1). Here this procedure is expected to produce the labeled fragment (X-P→)Manα1,4-GlcNα1,6-[3H]Ins with X-P-> being the substituent in question. The fragment was then either treated with JBAM or control incubated and finally dephosphorylated by HF, N-acetylated, and analyzed by paper chromatography. As can be seen in Fig. 6, A and B, the (X-P→)Manα1,4GlcNα1,6Ins fragment of M4 is JBAM-resistant, since successive treatment by acetylation, JBAM, and then HF generates Manα1,4GlcNα1,6Ins. The same had previously been found for CP2 (22). Thus, the difference between the head groups of M4 and CP2 cannot be explained by the presence or absence of an HF-sensitive substituent on Man1: both lipids have the same classical Manα-GlcNα ins carbohydrate core structure, they both contain HF sensitive groups on Man1 and Man3 (Fig. 1), but they migrate differently on TLC, and their non-dephosphorylated head groups elute differently on Dionex HPLC.
Man1 but lacks an HF-sensitive substituent on Man2. CP2 and M4 head groups were obtained from [3H]Man labeled Man1-P group on the GlcN in ones that have been documented in other organisms are the sensitive groups on GlcN, Man1, Man2, or Man3 or may be hypothesized that CP2 may contain either additional HF-sensitive substituent on Man2. If we assume that during HF treatment the EtN-Ps reaction intermediates lacking the HF-sensitive group on are hydrolyzed in a random order, we may expect to find some radioactivity contained in 1-cm wide strips was determined through scintillation counting. The different GPI-PLD and mild base treatment (Fig. 4A, lanes 3–6), suggesting that its lipid moiety consists of Ins-P-DAG. We previously reported that M4 is resistant to PI-specific phospholipase C (21). This finding, together with the GPI-PLD sensitivity, can be taken as an indication for the presence of an acyl moiety attached to the Ins of M4. We further released the (acyl→Ins)P-DAG moiety of M4 with HNO2 as described recently (41). As shown in Fig. 4B, the treatment of purified M4 by HNO2 produced a very hydrophobic species, which migrates very closely to M0, i.e. the GlcN(acyl)2→Ins-P-DAG accumulating in sec53 (Fig. 4B, lanes 7 and 9) (41). (As reported previously, the presence of GlcN on these early precursors does not significantly influence their migration in TLC, for discussion see Sipos et al. (41).) Partial deacylation of the M4-derived lipid moiety by NH3 produced PI lyso-PI (Fig. 4B, lane 10). This PI was compared with pG1, the PI species obtained by HNO2 treatment of protein-bound GPI anchors from the corresponding wt strain (Fig. 4B, lane 11). The comparison shows that M4 contains a PI moiety that migrates clearly less than pG1, whereas a lyso-PI of M4 migrates slightly more than the lyso-PI species generated by methanolic NH3 treatment of anchor peptides (Fig. 4B, lanes 10 and 12). Very similar results had been obtained previously when comparing protein-derived PI moieties with the PI moieties of M0 from sec53 and of CP2 from gpi8-1 (41). In addition we isolated from Δgpi7 the recently identified GPI intermediates that are obscured in TLC by PI and inositol phosphoceramides (Ref. 41, therein Fig. 6A), and we found that they are exactly the same as the corresponding intermediates from wt cells by all criteria (not shown). Thus, it seems that M4 and other GPI intermediates of Δgpi7 contain the same PI moiety as early and late GPI intermediates accumulating in other mutants or in wt cells, and we therefore conclude that the difference between CP2 and M4 is solely due the difference in their head groups.

Lack of Gpi7p Affects the in Vitro Biosynthesis of GPI Precursor Lipids—When yeast microsomes are incubated in the presence of UDP-[3H]GlcNAc, ATP, coenzyme A, GDP-Man, and tunicamycin, they generate labeled GPI intermediates as the only kind of labeled lipids (22, 47). Wild type microsomes make GPI intermediates up to CP2. Although a large array of incomplete intermediates is also generated, the pattern of labeled intermediates is fairly reproducible. When we used Δgpi7

Man3 while retaining EtN-P on Man2 or Man1. When such intermediates subsequently are treated with JBAM and then are dephosphorylated to completeness with HF, they should yield Man3-GlcN-Ins and Man1-GlcN-Ins fragments, respectively. For preliminary tests, CP2 head groups were first treated with HF for 12 h yielded substantial amounts of Man2-GlcN-Ins and Man1-GlcN-Ins (Fig. 6C), whereas the identical treatment performed with the head group of M4 only yielded Man1-GlcN-Ins but no Man2-GlcN-Ins (Fig. 6D). This result is compatible with the idea that Δgpi7 cells are unable to add an HF-sensitive group onto Man2 of the GPI core (Fig. 1). It also confirms the presence of an HF-sensitive group on Man1 of both M4 and CP2.

The Lipid Moieties of GPI Intermediates in Δgpi7 Are Normal—We looked for additional differences between M4 and CP2 by analyzing the lipid moiety of M4. M4 is sensitive to GPI-specific phospholipase D (GPI-PLD) and mild base treatment (Fig. 4A, lanes 3–6), suggesting that its lipid moiety consists of Ins-P-DAG. We previously reported that M4 is resistant to PI-specific phospholipase C (21). This finding, together with the GPI-PLD sensitivity, can be taken as an indication for the presence of an acyl moiety attached to the Ins of M4. We further released the (acyl→Ins)P-DAG moiety of M4 with HNO2 as described recently (41). As shown in Fig. 4B, the treatment of purified M4 by HNO2 produced a very hydrophobic species, which migrates very closely to M0, i.e. the GlcN(acyl)2→Ins-P-DAG accumulating in sec53 (Fig. 4B, lanes 7 and 9) (41). (As reported previously, the presence of GlcN on these early precursors does not significantly influence their migration in TLC, for discussion see Sipos et al. (41).) Partial deacylation of the M4-derived lipid moiety by NH3 produced PI lyso-PI (Fig. 4B, lane 10). This PI was compared with pG1, the PI species obtained by HNO2 treatment of protein-bound GPI anchors from the corresponding wt strain (Fig. 4B, lane 11). The comparison shows that M4 contains a PI moiety that migrates clearly less than pG1, whereas a lyso-PI of M4 migrates slightly more than the lyso-PI species generated by methanolic NH3 treatment of anchor peptides (Fig. 4B, lanes 10 and 12). Very similar results had been obtained previously when comparing protein-derived PI moieties with the PI moieties of M0 from sec53 and of CP2 from gpi8-1 (41). In addition we isolated from Δgpi7 the recently identified GPI intermediates that are obscured in TLC by PI and inositol phosphoceramides (Ref. 41, therein Fig. 6A), and we found that they are exactly the same as the corresponding intermediates from wt cells by all criteria (not shown). Thus, it seems that M4 and other GPI intermediates of Δgpi7 contain the same PI moiety as early and late GPI intermediates accumulating in other mutants or in wt cells, and we therefore conclude that the difference between CP2 and M4 is solely due the difference in their head groups.

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Microsomes of W303 wild type or Δgpi7 were incubated with 6 μCi of UDP-\(^{3}H\)GlcNac, GDP-Man, tunicamycin, and ATP for 1 h at 37 °C as described (22). The glycolipid products were extracted and then run on TLC with solvent 2. The extract in lane 3 was first treated with JBAM. On the basis of the preceding analysis the band denoted with an asterisk can be presumed to be an M4 derivative in which Man4 has been removed.

Microsomes, they reproducibly made all the normal intermediates down to a band comigrating with M4 of \(^{3}H\)Ins-labeled Δgpi7 cells, but they consistently failed to make CP2 (Fig. 7). When the labeled lipid extract was treated with JBAM, most of the band comigrating with M4 was shifted to a less hydrophilic position, much in the same way as seen for \(^{3}H\)Ins-labeled M4 (Fig. 2, lanes 1 and 2). Thus, the M4 accumulation of Δgpi7 can be reproduced in vitro. This result implies that the Gpi7p present in wt microsomes is functional in vitro.

Characterization of Gpi7p—Gpi7p was characterized using affinity purified rabbit antibody made against the N-terminal, hydrophilic portion of GPI7 (Fig. 3B). As shown in Fig. 8A, the antibody recognized a heterogeneously glycosylated 208-kDa protein, the estimated molecular mass of various glycoforms ranging, after heavy exposure, from about 130 to 230 kDa (Fig. 8A, lane 2). The predicted mass of the protein before and after removal of the signal sequence is 94,832 and 92,207 Da, respectively. In glycosylation mutants losch1/mnn1 or mnn9 which are totally or partially deficient in the elongation of N-glycans, the Golgi, Gpi7p has an estimated mass of 108 and 115 kDa, respectively (Fig. 8A, lanes 1 and 7). pmi40 has a ts deficiency in Man biosynthesis that is partial at 24 °C (48). In pmi40 grown at 24 °C the average mass of Gpi7p is around 150 kDa (Fig. 8A, lane 6). (This suggests that full elongation of N-glycans is not necessary for Gpi7p function since, when shifted from 24 to 37 °C, pmi40 cells are able to make CP2 (23)). Tunicamycin treatment of wt or pmi40 cells resulted in the appearance of a single, relatively sharp band of an apparent molecular mass of about 83 kDa (Fig. 8A, lanes 3 and 5). The protein could also be deglycosylated to an apparent molecular mass of 86 kDa by treatment with endoglycosidase H (not shown). All these data concurrently indicate that Gpi7p contains several N-glycans that are heavily elongated in the Golgi but contains no or only few O-glycans. In the cell lysate Gpi7p was rapidly degraded by an endogenous protease which, however, could be inhibited by 10 mM EDTA. Gpi7p is associated with membranes since it could be sedimented by ultracentrifugation of lysates at 100,000 × g for 15 min at 4 °C, and microsomal pellets were processed for SDS-PAGE. Cells in lanes 3 and 5 had been grown in 20 μg/ml tunicamycin (Tun) for 90 min. B–D, exponentially growing W303 cells were broken with glass beads using the buffers indicated under “Experimental Procedures,” and cell wall debris was removed by centrifugation at 600 × g. B, aliquots of cell lysate were incubated for 30 min at 0 °C with 0.5 mM NaCl, 0.8 M urea, 1% Triton X-100 (TX-100), 0.1 mM Na2CO3, pH 11, or 1% SDS. Subsequently membranes were sedimented by ultracentrifugation to get supernatant (S) and pellet (P) fractions. C, cell lysate was sedimented at 13,000 × g for 15 min, and the membrane pellet was thoroughly resuspended and digested with 10 or 25 μg/ml proteinase K (prot K) at 0 °C for 20 min in the presence or absence of 0.5% Triton X-100. D, cell lysates were subjected to differential centrifugations at 8,000 and 100,000 × g. These centrifugations generated pellet P8 containing ER, PM, and vacuolar membranes and pellet P100 which contains Golgi membranes. The 8,000 × g supernatant was also precipitated with trichloroacetic acid (TCA). In all panels the lanes contain material derived from 1 × 106 cells except for lanes 1 and 7 of A which contain 0.3 × 106.
may be underestimated due to ongoing proteolytic degradation during the 100,000 × g spin. Therefore the supernatant of the 8,000 × g spin was split whereby proteins were immediately precipitated with trichloroacetic acid in one half, and the other half was pelleted at 100,000 × g. Gpi7p was exclusively found in the 8,000 × g pellet (P8) and thus is associated with either the ER, the vacuole, or the PM but not with the Golgi. As shown in Fig. 9A low amounts of proteinase K (prot K) exactly as described (65) except that the EDTA concentration was raised to 20 mM. In lane 6, Triton X-100 was added to 1% B, W303 cells were treated with zymolyase 20T at the indicated concentrations. C, W303 or Δgpi7 cells were either lysed directly or after having been incubated for 30 or 60 min at 10 A260/ml in the presence of cycloheximide (200 μg/ml) or tunicamycin (Tm, 20 μg/ml). Cells were lysed by boiling in sample buffer and processed for SDS-PAGE and Western blotting with antibodies against Gpi7p, Gas1p, or Wbp1p.

Fig. 9. Gpi7p is localized at the cell surface. Cells in the early exponential phase growing at 30°C in YPD were used. A, intact W303 cells were treated with cysteamine chloride and then treated with the indicated concentrations of proteinase K (prot K) as described (85) and the EDTA concentration was raised to 20 mM. In lane 6, Triton X-100 was added to 1% B. W303 cells were treated with zymolyase 20T at the indicated concentrations. C, W303 or Δgpi7 cells were either lysed directly or after having been incubated for 30 or 60 min at 10 A260/ml in the presence of cycloheximide (200 μg/ml) or tunicamycin (Tm, 20 μg/ml). Cells were lysed by boiling in sample buffer and processed for SDS-PAGE and Western blotting with antibodies against Gpi7p, Gas1p, or Wbp1p.

TABLE I
Quantitation of GPI anchor lipids

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<tr>
<th>pG1</th>
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<tr>
<td>X2180</td>
<td>24.2</td>
<td>61.2</td>
</tr>
<tr>
<td>Δgpi7</td>
<td>23.8</td>
<td>63.9</td>
</tr>
<tr>
<td>Δgpi7</td>
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<td>19.3</td>
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</tbody>
</table>

Deletion of Gpi7 Alters GPI Protein Transport and Remodeling—We previously reported on the accumulation of the immature 105-kDa ER form of Gas1p in gpi7 mutants. We therefore investigated GPI protein transport in Δgpi7. Indeed, by pulse-chase experiments we found that the maturation of GPI proteins Sag1p and Gas1p was slowed 2–3-fold as compared with wt cells, whereas the maturation of carboxypeptidase Y proceeded with normal kinetics (not shown). This indicates that the transport of GPI proteins in Δgpi7 is specifically retarded. Nevertheless, in rich media Δgpi7 cells grow at roughly the same rate as wt cells. They also incorporate [3H]Ins with the same efficiency as wt cells.

The lipid remodeling of GPI anchors is significantly altered in Δgpi7. As seen in Table I, the proportion of ceramides (pC1 and pC2) in anchor peptides from Δgpi7 is drastically decreased, whereas the fraction of DAG-containing lipids (pG1) is correspondingly increased. It should be noted that at the time of analysis, i.e. 75 min after addition of [3H]Ins, the relative amounts of mild base-sensitive and mild base-resistant anchors are no longer changing and represent the steady state proportion of these two anchor types (41, 52). It is important to realize that pG1 also represents a remodeled form of the anchor lipid in which a long chain fatty acid has replaced the original fatty acid present in sn-2 of the glycerol of the CP (Fig. 1). It thus appears that the relative decrease of ceramide remodeling goes along with a compensatory increase in DAG remodeling. A relative reduction in ceramide remodeling was also observed when we compared the efficiency of [3H]Ins and [3H]dihydrophosphoglycerol ([3H]DHS) incorporation into GPI proteins. As can be seen in Fig. 10, the ratio of [3H]DHS/[3H]Ins incorporation into proteins is much higher in wt than in Δgpi7 (Fig. 10, lanes 1–4). The lack of incorporation of [3H]DHS in Δgpi7 cannot be explained by an increase of the endogenous production of DHS in Δgpi7, since the difference between wt and Δgpi7 persists, even when all endogenous DHS biosynthesis is blocked by myriocin (Fig. 10, lanes 5 and 6). The defect in
remodeling seems to be affecting mostly the maturation processes in the Golgi and/or PM (Golgi/PM remodeling) since, as shown in Fig. 10, lanes 7–10, the ratio of [3H]DHS/[3H]Ins incorporation into proteins in the Δgpi7/sec18 double mutant was the same as in sec18. Also, when using stringent conditions under which one observes only ER or only Golgi/PM remodeling (24), remodeling in the ER appeared relatively normal, whereas remodeling in the Golgi/PM was reduced (Fig. 10, lanes 11–14, 11′, and 12′). The relatively low amount of pC2 in anchor lipids of Δgpi7 (Table I) may be a consequence of this relative deficiency of Golgi/PM remodeling, since pC2 type anchors are only generated by the Golgi/PM but not the ER remodelase (24, 41). The relationship between the specific retardation of GPI protein transport, reduced Golgi/PM remodeling, and increased remodeling toward pG1 is for the moment unclear.

**DISCUSSION**

Yeast and mammals contain the same GPI carbohydrate core structure. This suggests that the GPI anchoring pathway has been established early in evolution and has rigorously been conserved in widely diverging organisms. On the other hand, the side chains added to this core as well as the lipid moieties of the anchor tend to vary a lot between different species (1). The GPI anchors of *S. cerevisiae* contain two types of side chains as follows: one or two mannoses are linked to Man3 (53) and an EtN-P side chain is linked to Man1. Both side chains are already present on the precursor lipid CP2 (22, 23). These two side chains are also found in some vertebrates, including mammals, and possibly in *Dictyostelium discoideum* (1), suggesting that not only the GPI core structure but also certain kinds of side chains have been invented and conserved since early times of evolution. Here we present evidence for yet a further, possibly conserved HF-sensitive substituent on CP2 which is attached to Man2. So far, the only side chain attached to Man2 reported in the literature is EtN-P. EtN-P was found by mass spectrometry on 15% of anchors of human erythrocyte cholinesterase and 3% of bovine liver 5′-nucleotidase (44, 46). Partial acid hydrolysis has also indicated an HF-sensitive substituent on Man2 in 40% of CD52-II (45). Analysis of the ethanolamine/Ins ratio in GPI anchors of porcine renal membrane dipeptidase and of human placental alkaline phosphatase yielded values of 2.5 and 2.4, suggesting the presence of EtN-P on Man2 in 50 and 40% of their anchors, respectively (54, 55). However, no such side chain was detected in other mammalian GPI proteins such as rat brain Thy-1 glycoprotein (56) or hamster scrapie prion (57). It may be that in many studies part or all of EtN-P side chains on Man2 and Man1 were hydrolyzed by an unspecified phosphodiesterase during the purification of the respective GPI proteins and preparation of anchor peptides using Pronase. Such a phosphodiesterase activity may explain why we failed to detect any EtN-P side chain on GPI-anchored yeast proteins in the past (53), although we now have firm evidence for the presence of an HF-sensitive substituent on Man1. The chemical nature of the side chain on Man2 of CP2 remains to be determined. The presence of an HF-sensitive side chain on Man2 of CP2 has its parallel in human cells. Indeed, there is evidence for an HF-sensitive group on Man2 of H8, the most polar GPI lipid of HeLa cells (58). The EtN-P side chains on Man1 and Man3 being conserved between mammalian organisms and yeast, it appears reasonable to speculate at this point that the analogy between mammalian and yeast anchors may extend to the substituent on Man2, *i.e.*, that also the side chain on Man2 of yeast GPI structures may consist of an EtN-P and that this EtN-P may be present on some mature GPI proteins of *S. cerevisiae*.

Δgpi7 cells are hypersensitive to Calcofluor White and hence have some difficulty in constructing their cell walls. Several reasons can be envisaged. (i) The side chain on Man2 may be important for the interaction of CPs with the transamidase complex and for their efficient transfer onto proteins. Recent data show that a small reduction of Gpi8p renders transamidase activity rate-limiting. The synthetic effect of gpi7 mutations with gpi8 mutations suggests that deletion of GPI7 may have a similar effect. A decreased transamidase activity may particularly affect the anchoring of certain GPI proteins that have a low affinity for the transamidase even though the global rate of GPI biosynthesis and [3H]Ins incorporation into proteins of Δgpi7 is not grossly reduced. Thus it is conceivable that some GPI proteins important for cell wall architecture are lacking in Δgpi7. (ii) The side chain on Man2 may serve as an attachment point for the covalent linkage of β1,6-glucans to the anchor moiety of cell wall proteins although a recent analysis of the linkage region between the GPI anchor remnant and β1,6-glucans rather showed a direct glycosidic linkage between Man1 and the β1,6-glucan (6). (iii) The side chain may serve as a recognition signal for enzymes or proteins that facilitate the packaging of GPI proteins into vesicles, for remodelases that

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exchange their lipid moieties, or for hydrolases or transglycosidases that remove parts of the GPI anchor of cell wall proteins and hop the GPI remnant onto β1,6-glucans (4, 5).

Our data further show that ceramide remodeling in the Golgi/PME is significantly reduced in Δgpi7, whereas remodeling toward pG1 is increased whereby it is not clear if pG1 remodeling is increased because ceramide remodeling is decreased or if ceramide remodeling is decreased because pG1 remodeling is increased. Moreover, the relationship of the alteration of GPI remodeling with the other phenotypic changes of Δgpi7 can be explained in several ways. (i) Previous studies showed that remodeling toward pG1 occurs in the ER and that retention of GPI proteins in the ER in secretion mutants maintains a high pG1/pC1 ratio on these proteins (41). Thus, if the substituent on Man2 of GPI anchors is important for efficient packaging of GPI proteins into transport vesicles, the delay in export of GPI proteins out of the ER may give the ER remodelase generating pG1 prolonged access to the GPI proteins and may thus cause a relative increase of pG1. (ii) The side chain on Man2 may serve as a recognition signal for Golgi/PME remodelase. (iii) We also considered the possibility that Gpi7p itself may be a Golgi/PME remodelase. This latter hypothesis would not directly explain why Δgpi7 cells cannot attach the HF-sensitive substituent onto Man2 and would imply that the addition of this side chain somehow is directed by the prior attachment of a ceramide moiety. This, however, is clearly not the case, since CP2 also contains the HF-sensitive side chain on Man2, although its lipid moiety consists of DAG (23). Thus we believe that the reduced Golgi/PME remodeling of GPI proteins in Δgpi7 is secondary to the lack of a substituent on Man2.

Our previous data suggested that CP2 can be transferred to proteins (23), and our working hypothesis until recently assumed that CP2 represents the GPI lipid used for GPI anchoring also by normal cells that do not accumulate this lipid (“CP2 hypothesis”). By consequence we would have predicted that all the enzymes required for the elaboration of CP2 are localized in the ER. Paradoxically, the subcellular fractionation experiments and protease treatment of intact spheroplasts strongly suggest that the bulk of Gpi7p resides at the cell surface (Fig. 9, A and B). Moreover, although we recently succeeded in demonstrating the presence of an HF-sensitive group on Man1 of immature ER forms of GPI proteins, we presently lack the tools to look for such a group on Man2. Thus, the so far available data raise a doubt whether it is CP2 which is added to GPI proteins in the ER, and we therefore are presently considering the possibility that other GPI lipids than CP2 are the physiological substrate of the ER transamidase. In fact, neither CP2 nor M4 can be detected in wt cells. It therefore seems possible that under physiological conditions cells add M4 to GPI proteins (“M4 hypothesis”) and that CP2 is elaborated only in mutants in which M4 cannot be transferred to proteins, spills out of the ER, and reaches the PM. It is noteworthy that Δgpi7 incorporates [3H]Ins at a normal rate into proteins suggesting that the transamidase is perfectly able to transfer M4. Thus, the side chain on Man2 may normally not be added to GPI proteins or only be added after GPI proteins arrive at the surface. The M4 hypothesis, however, does not explain why M0 and M4 accumulate in Δgpi7, whereas M0, M4, and CP2 remain undetectable in wt cells (Fig. 2, lanes 1 and 2) or why gpi8-1, deficient in the transfer of GPIs onto proteins, accumulates CP2 (19, 21). It also fails to explain the delayed maturation of GPI proteins and the reduced rate of GPI remodeling observed in Δgpi7. To save the M4 hypothesis, the accumulation of GPI intermediates in Δgpi7 could be rationalized by assuming that the substituent on Man2 serves to mark supernumerary GPIs for degradation, but also this assumption does not explain the observed accumulation of CP2 in gpi8. Thus, although our results raised the possibility that M4 is the physiological GPI lipid for GPI anchoring, this M4 hypothesis leaves many results unexplained and the data are more easily explained by our original CP2 hypothesis. For one, the synchronous accumulation of M4 and CP2 in all our gpi8 mutants argues that M4 is not a better substrate for the transamidase than CP2. CP2 may physiologically be produced by the small amount of Gpi7p in the ER (Fig. 9C). Alternatively, it is conceivable that M4 is transported from the ER to the PM, is converted there to CP2, and is then transported back to the ER by some not yet elucidated mechanism. In this context it is noteworthy that the biosynthesis of GPIs by wt microsomes in vitro produces CP2 in good yield, i.e. the in vitro system adds the substituent on Man2. This in vitro system does not contain cytosol nor GTP and hence should not support vesicular transport from ER- to Golgi-derived microsomes (59). It is possible, however, that GPI lipids are transported between microsomes or membrane fragments by means of lipid transfer proteins or through direct contact between membranes. It also can be envisaged that juxtaposition of membranes allows enzymes present in one membrane to work on lipids in another membrane. The same mechanisms may also operate in intact cells. Clearly the identity of the physiological GPI lipid substrate of the transamidase will have to be established by further experiments.

Homology searches show that two ORFs of S. cerevisiae are related to GPI7, MCD4 (= YKL165c), and YLL031c. They belong to a novel gene family comprising for the moment the nine members shown in Table II which, based on the many predicted transmembrane domains, were previously classified as putative permeases (60). Pairwise alignment allows us to group

### Table II

<table>
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<tr>
<th>Gene</th>
<th>Homologue</th>
<th>Organism</th>
<th>Score</th>
<th>PDB</th>
<th>Subfamily</th>
</tr>
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<td>29</td>
<td>39</td>
<td>Gpi7, GPI7</td>
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<tr>
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<td>22</td>
<td>26</td>
<td>GPI7</td>
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<tr>
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<td>19</td>
<td>16</td>
<td>GPI7</td>
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<tr>
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<td>9.</td>
<td>(2879870) S. cerevisiae</td>
<td>16</td>
<td>14</td>
<td>14</td>
<td>12</td>
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</table>

Homologous sequences were compared by ClustalW at the European Bioinformatics Institute (www2.ebi.ac.uk/clustalw/). The aligned scores for pairwise alignments are reported; a high score indicates high homology. ORFs that have not received gene names are indicated by their chromosomal denominator (S. cerevisiae) or the NCBI protein identification number (PID). Subfamilies are separated by blank lines. Alignments within a given subfamily are in bold. S. cerevisiae; S. pombe.
them into three subfamilies of more closely related ORFs. All nine ORFs predict membrane proteins of about 100 kDa having an N-terminal signal sequence, a hydrophilic N-terminal part, and multiple transmembrane domains in their C-terminal half. mcd4 mutants were obtained in a screen for cells deficient in the cell cycle controlled polarization of growth, a phenotype also generated by mutations in the exocyst or in N-glycosylation (61). The subfamilies typified by GPI7 and YLL031c are more closely related to each other than to the MCD4 subfamily. All nine family members contain two conserved motifs at about the same position in the hydrophilic N-terminal domain, namely HXLGXXXXXG and DHGXXVXXGH. These motifs are also found in two EST clones from human cDNA that have high homology to MCD4 (NCBI PID 177947 and 1765215, ClustalW alignments giving aligned scores of 46 and 35). Very interestingly, by a reiterated Psi Blast search at the National Center of Biotechnology Information (NCBI) (62) one can find a highly significant homology of all three subfamilies with a large family of phosphodiesterases. The large majority of these homologous sequences encode mammalian cell-surface proteins classified as alkaline phosphodiesterase I, nucleotide pyrophosphatase, or alkaline phosphatase. The homology comprises a region of about 220–240 amino acids in the N-terminal hydrophilic part of GPI7, YLL031c, and MDC4. The homology of GPI7 in this region with mammalian and plant phosphodiesterases amounts to 17–18% identity and 30–34% similarity and comprises a motif PTXXTXXXG which is common to bacterial, viral, plant, and mammalian phosphodiesterases. This homology may suggest that Gpi7p itself is the transerase adding the phosphodiester linker substituent on Man2. In this context it is interesting to note that the En-N-P on Man3 of the GPI anchor has been shown to be transferred by transesterification using phosphatidylethanolamine as donor of En-N-P (63, 64). Mutants in YLL031c also accumulate abnormal GPI intermediates which on TLC have about the same mobility as M4 suggesting that YLL031c is similarly involved in adding En-N-P.2 Thus, it is conceivable that not only the GPI7 subfamily but also other subfamilies are involved in the transfer of En-N-P onto the GPI core structure. However, the transesterification activity of Gpi7p will have to be shown directly before one can exclude that the primary function of Gpi7p is to generate some signal from the cell wall which regulates GPI protein transport and remodeling as well as side chain addition to structures with GPI anchor side chains.

It is interesting that subfamily members belonging to different species are more closely related to each other than family members belonging to a single species. This can be seen when comparing the pairwise alignment scores among the three ORFs of S. cerevisiae or the three ORFs of Schizosaccharomyces pombe with the scores among subfamily members (Table II). In evolutionary terms this suggests that the divergence of these three subfamilies occurred earlier than the separation of the lineages leading to S. cerevisiae, S. pombe, and Caenorhabditis elegans. This implies that the HF-sensitive group on Man2 is of very ancient origin. GPI7 bears no resemblance with PIG-F, a mammalian gene encoding for a highly hydrophilic membrane protein involved in the addition of En-N-P to Man3 (10). The exact role of PIG-F has not yet been elucidated.

It will be interesting to find the human homologues of GPI7. It may be that this gene, as in S. cerevisiae, plays a more dispensable role in GPI anchoring than the enzymes involved in the elaboration of the carbohydrate core structure such as for instance PIG-A/PIGP3/CWH6/SPT47 (7). Thus, although deficiencies in PIG-A are only acquired by somatic cells, deficiencies in the human GPI7 homologue may be transmissible through the germ line as well.
GPI Anchor Side Chains

Deletion of GPI7, a Yeast Gene Required for Addition of a Side Chain to the Glycosylphosphatidylinositol (GPI) Core Structure, Affects GPI Protein Transport, Remodeling, and Cell Wall Integrity

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