YLL031c Belongs to a Novel Family of Membrane Proteins Involved in the Transfer of Ethanolaminephosphate onto the Core Structure of Glycosylphosphatidylinositol Anchors in Yeast*

Isabelle Flury, Abdellah Benachour, and Andreas Conzelmann‡

From the Institute of Biochemistry, University of Fribourg, CH-1700 Fribourg, Switzerland

MCD4 and GPI7 are important for the addition of glycosylphosphatidylinositol (GPI) anchors to proteins in the yeast Saccharomyces cerevisiae. Mutations in these genes lead to a reduction of GPI anchoring and cell wall fragility. *Gpi7* mutants accumulate a GPI lipid intermediate of the structure Manα1-2Manα1-2Manα1-6[Manα1-2CH2-NH2]2PO4-→Manα1-2Manα1-2Manα1-6Manα1-4GlcNa1-6[acetyl-]inositol-PO4-lipid, which, in comparison with the complete GPI precursor lipid CP2, lacks an HF-sensitive side chain on the α1-6-linked mannose. In contrast, mcd4-174 accumulates only minor amounts of abnormal GPI intermediates. Here we investigate whether YLL031c, an open reading frame predicting a further homologue of *GPI7* and *MCD4*, plays any role in GPI anchoring. YLL031c is an essential gene. Its depletion results in a reduction of GPI anchor addition to GPI proteins as well as to cell wall fragility. YLL031c-deleted cells accumulate GPI intermediates with the structures Manα1-2Manα1-2Manα1-6[Manα1-2CH2-NH2]2PO4-→Manα1-4GlcNa1-6acetyl-]inositol-PO4-lipid and Manα1-2Manα1-2Manα1-6Manα1-4GlcNa1-6[acetyl-]inositol-PO4-lipid. Subcellular localization studies of a tagged version of YLL031c suggest that this protein is mainly in the ER, in contrast to Gpi7p, which is found at the cell surface. The data are compatible with the idea that YLL031c transfers the ethanolaminephosphate to the inner α1-2-linked mannose, i.e. the group that links the GPI lipid anchor to proteins, whereas Mcd4p and Gpi7p transfer ethanolaminephosphate onto the α1-4- and α1-6-linked mannosides of the GPI anchor, respectively.

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The abbreviations used are: GPI, glycosylphosphatidylinositol; CFW, Calcofluor White; CP2, complete precursor 2; EtN-P, ethanolaminephosphate; Ins, myo-inositol; JBAM, jack bean α-mannosidase; nt, nucleotide(s); SFH, short flanking homology; WT, wild type; PCR, polymerase chain reaction; bp, base pair(s); ER, endoplasmic reticulum.

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

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Additional information is available in the full text of the article.
Man2, Man3, and Man4 are used here to designate the glycolipid CP2. Relevant cleavage procedures are indicated. Man1, separation of YLL031c was achieved by the insertion of the was from Sigma.

(Mikrobiologisches Institut, ETH Zürich, Switzerland), respectively. A Yap3p and Cwp1p were kindly donated by Dr. Y. Bourbonnais (Univer-
tidase Y from Molecular Probes, Inc. (Eugene, OR). Rabbit antibodies to
dida), Calcofluor White (CFW) from Sigma, and antibodies to carboxypep-
GGCAACGCCGctcttggcctcctctag-3

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Previously described procedures were used to label cells with [2-3H]Ins and for lipid extraction (20). In vitro biosynthesis of GPIs with microsomes was performed as described previously (4), with the exception that the spheroplasts were sometimes prepared using Quantzyme ylg instead of zymolyase-20T. Desalted lipid extracts were analyzed by ascending TLC using solvent 1 (CHCl₃/CH₃OH/H₂O, 10:10:3, v/v/v) or solvent 2 (chloroform/methanol/0.25% KCl, v/v/v). Radioactivity was detected and quantitated by two-dimensional radiochromatography (LB 2842; Berthold AG, Regensdorf, Switzerland). Thereafter, TLC plates were sprayed with ENHANCE and exposed to film (X-Omat; Eastman Kodak Co.) at −80 °C.

Soluble head groups were obtained from purified radiolabeled glycolipids through limiting methanolic NH₃ deacylation (21) followed by PI-PLC treatment, for which the lipid extracts were dissolved in 20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 20% 1-propanol. Incubations with PI-PLC were for 16 h at 37 °C. Nonhydrolyzed GPIs were removed by butanol extraction. The water-soluble head groups were treated with JBAM (0.5 units) or A. saitoi α-mannosidase (5 microunits) as described (22). HF dephosphorylation was done as described (23). The generated fragments were analyzed by paper chromatography in methylethylketone/pyridine/H₂O (20:12:11) as described (23). Before paper chromatography the products were N-acetylated and desalted over mixed bed ion exchange resin AG-501-X8 (Bio-Rad). Radiolabeled chromatography standards were obtained as described (8).

RESULTS
Characterization of YLL031c—YLL031c was disrupted in a diploid strain by the replacement of most of the open reading frame by the KanMX kanamycin resistance gene. This deletion strain was constructed because in the YLL031c deletion strain produced in the EUROFAN project, part of YLL030c has been deleted along with YLL031c (24). Sporulation and dissection of tetrads yielded in all cases two growing colonies per tetrad. None of these colonies were kanamycin-resistant, indicating that YLL031 is essential. A partial depletion of YLL031 was achieved by the insertion of the glucose-repressible GAL1,10 promoter immediately upstream of the genomic YLL031c. When such cells, growing in liquid culture, were shifted from galactose to glucose, therefore allowing for the partial depletion of the YLL031c protein, their doubling time increased, after 24 h, to 3.5 h, and cells thereafter continued to grow at this rate, whereas WT cells grew on glucose with a doubling time of 1.3 h. When the same experiment was done in a Δgpi7 background, the doubling time of YLL031c-depleted cells, after 24 h on glucose, had risen to 7.7 h, while Δgpi7 mutants grew as fast as WT cells. Thus, the deletion of GPI7 synthetically enhances the growth retardation caused by depletion of YLL031c. The reduced growth rate of YLL031c-depleted cells could also be observed on YPD plates, and this effect was significantly enhanced by the presence of CFW at a concentration that did not affect growth of WT cells but totally blocked the growth of Δgpi7 cells (Fig. 2). This result indicates that partial depletion of YLL031c leads to cell wall fragility, a phenotype that is commonly observed in mutants affected in the GPI biosynthesis pathway.

Depletion of YLL031c Leads to the Accumulation of Immature GPI Proteins—If Gas1p does not receive a GPI anchor in the ER, it fails to be transported to the Golgi (25), because, as shown by in vitro experiments using a vesicle budding assay, nonanchored Gas1p is not packaged into COPII-coated transport vesicles budding off the ER (26). Therefore, the characteristic mass increase due to elongation of N- and O-glycans in the Golgi does not take place, and the maturation of the immature 105-kDa ER form into the mature 125-kDa form is delayed. GPI anchoring deficiencies also can lead to the relative depletion of the mature forms of Gas1p and other GPI proteins (27). Using Western blotting and antibodies to detect well characterized GPI proteins, we could observe a significant depletion of mature GPI proteins upon depletion of YLL031c. As can be seen in Fig. 3, while in WT cells the carbon source affects the amount of Gas1p only moderately, cells partially depleted of YLL031c by growth on glucose contain much less of the mature 125-kDa form of Gas1p than cells grown on galactose. This phenomenon is even more drastic when YLL031c depletion occurs in a Δgpi7 background. Other GPI proteins such as Yap3p and Cwp1p are also affected. Although the details of the

![Fig. 2. Characterization of YLL031c.](Image)

![Fig. 3. Depletion of YLL031c leads to a depletion of mature and accumulation or depletion of immature forms of GPI proteins.](Image)
biosynthesis of these proteins have not been reported and part of their mature forms gets covalently attached to the cell wall, the mass of their ER forms can be inferred from the peptides accumulating in sec18, a mutant that blocks the protein traffic from ER to Golgi at 37 °C (28, 29). YAP3 predicts a 60-kDa translation product, but upon shift to 37 °C, sec18 cells rapidly accumulate lower molecular weight forms (Fig. 3). Similar forms also accumulate upon depletion of YLL031c, suggesting a maturation defect of these proteins due to a delay in GPI anchor addition. Cwp1p is a cell wall protein that can be released as soluble 55–60-kDa protein by β-glucanase (30, 31).

There are also intracellular, detergent-soluble forms of Cwp1p of 48 and 58 kDa, and these forms are drastically increased when the secretory pathway is blocked for 2 h at 37 °C in sec18 (Fig. 3). In YLL031c-depleted cells, these detergent-soluble forms are severely diminished. This also can be taken as evidence for some disturbance of GPI protein maturation, although in this case the protein does not seem to accumulate but may be rapidly degraded. In contrast, YLL031c-depleted cells do not accumulate the typical ER proform p1 of carboxypeptidase Y, a vacuolar hydrolase, nor do they show a thinning of mature carboxypeptidase Y (Fig. 3). This suggests that the maturation defect of YLL031c-depleted cells affects only GPI proteins.

Depletion of YLL031c Leads to the Accumulation of Abnormal GPI Lipids—When WT cells are metabolically labeled with [methyl-3H]inositol ([3H]Ins), their lipid extracts contain various forms of labeled phosphatidylinositol and inositolphosphorylceramide, but the GPI intermediates remain undetectable. GPI intermediates have, however, been found to accumulate in several gpi mutants. As can be seen in Fig. 4, A and B, YLL031c-depleted cells accumulate the abnormal lipids 031a and 031b, which are present neither in WT nor in previously described gpi mutants. Lipid 031a migrates quite differently from M2*, the most polar lipid accumulating in M2*, the most polar lipid accumulating in (Fig. 4). Several intermediates have, however, been found to accumulate in several gpi mutants. As can be seen in Fig. 4, and 031b, which are present neither in WT nor in previously described gpi mutants. Lipid 031a migrates quite differently from M2*, the most polar lipid accumulating in gpi10–1, which has the structure Man1–6[Ins–(CH2)2–PO4]–Man1–4GlcN1–6[acyl]–Ins–PO4–lipid (Fig. 4A, lanes 3 and 4) (4). The more polar lipid 031b has a lower Rp than M2* but a higher Rp than M4, the GPI lipid that accumulates in Δgpi7 cells and has the structure Manα1–2[Ins–(CH2)2–PO4]–Manα1–2Manα1–6[Ins–(CH2)2–PO4]–Man1–4GlcN1–6[acyl]–Ins–PO4–lipid (Fig. 4A, lanes 3 and 4) (8). A block of GPI biosynthesis can also be observed in vitro with YLL031c-depleted microsomes. WT microsomes make the complete GPI precursor CP2 irrespective of the carbon source on which cells have been grown (Fig. 4C, lanes 8 and 9). In contrast, YLL031c-depleted microsomes make considerably less CP2 but instead accumulate a less polar lipid that also has a higher Rp than the M4 made by Δgpi7 microsomes (Fig. 4C, lanes 3, 4, 10, and 11). This in vitro generated lipid comigrates with in vivo made 031b (Fig. 4C, lanes 2 and 4).

031a and 031b are less polar than M4, suggesting that they have smaller head groups and are earlier intermediates of GPI biosynthesis than M4. It therefore is not unexpected that the accumulation of 031b is epistatic to the accumulation of M4 both in vitro and in vivo. Indeed, the combination of Δgpi7 with YLL031c depletion strongly reduces the accumulation of M4 and increases the accumulation of 031b (Fig. 4, A, lane 6, and C, lanes 5 and 6). Since the depletion of YLL031c is only partial, it is understandable that in vitro there still is residual biosynthesis of CP2 or M4 in single or double mutants, respectively (Fig. 4C, lanes 4, 6, and 11). The severely affected med4–174 mutant has been reported to accumulate only trace amounts of abnormal [3H]Ins-labeled lipids (10). We find the same if Med4p is depleted using the repressible GAL1,10 promoter (Fig. 4A, lane 5).

**Fig. 4.** YLL031c-depleted cells accumulate at least two abnormal GPI lipids. A and B, exponentially growing cells were radiolabeled with [3H]Ins at 37 °C (4 μCi/1–2 × 105 cells), and desalted lipid extracts were analyzed by TLC using solvent 1 (A) or solvent 2 (B) and fluorography. C, for lanes 1 and 2, cells were labeled with [3H]Ins (I) and processed as described for A. Lanes 3–14, microsomes were prepared from exponentially growing cells and were incubated with 3 or 6 μCi of UDP-[3H]GlcNac (GN), GDP-Man, tunicamycin, CoA, and ATP for 1 h at 30 °C (lanes 3–7) or 2 h at 30 °C (lanes 8–14) as described (4), and lipid extracts were analyzed by TLC using solvent 1 and fluorography. Extracts in lanes 12 and 14 were first treated with JBAM. FBY1102 and FBY1106 were grown in medium containing either galactose (G) or glucose (D). MIP1C, mannosyl-GlcN-lipid. We infer that the substituent on man1 of M2* of the structural characterization of lipids. O31a and O31b were purified by preparative TLC. Their structural characterization yielded the following information. Both lipids are effectively cleaved by GPI-specific phospholipase D, indicating that they are GPIs. They are totally resistant to bacterial, phosphatidylinositol-specific phospholipase C, suggesting that they contain a protecting acyl group on the Ins. Their label becomes entirely hydrophilic upon deacylation by mild alkaline hydrolysis. Analysis of the hydrophilic head group of lipid 031b indicates that it contains a Manα1–GlcN–Ins core structure (Fig. 5A), that two or three of its mannoses can be removed by JBAM (Fig. 5C), whereas the same enzyme removes all four mannoses from the HP′ dephosphorylated head group (Fig. 5D). If the HP′-treated core structure is treated with Aspergillus satoy a-amylase, a linkage-specific exo-a-mannosidase cleaving α1′–2 bonds, one obtains Manα1–GlcN–Ins (Fig. 5B). These data strongly suggest that the bulk of lipid 031b has the structure Manα1–2Manα1–2Manα1–6[Ins–(CH2)2–PO4]–Manα1–4GlcN1–6[acyl]–Ins–PO4–lipid. We infer that the substituent on Man1 consists of EtN-P, because this substituent has been demonstrated to be present on Man1 of M2* of the gpi10–1 mutant (4). The sensitivity of the in vitro made lipid 031b to JBAM (Fig. 4C, lanes 11 and 12) is in agreement with the
spheroplasts were prepared with Quantazyme, both YLL031c and its previously reported susceptibility to zymolyase (8). When detected on any blot in this experiment (not shown) because of surface protein and as described before (8). Gpi7p could not be reduced by increasing the concentration of JBAM. This suggests the presence of a lipid having the structure Manα1–2Manα1–2[NH2–(CH2)2–PO4–] Manα1–6Manα1–4GlcNα1–6[acyl–]Ins–PO4–lipid, which we call 031b in the following. Based on its comigration with 031b on TLC we assume that 031b also carries no more than one EtN-P group.

Analysis of the hydrophilic head group of lipid 031a indicates that it contains a Manα2–GlcNα–Ins peak in Fig. 5C could not be reduced by increasing the concentration of JBAM. This suggests the presence of a lipid having the structure Manα1–2Manα1–2[NH2–(CH2)2–PO4–] Manα1–6Manα1–4GlcNα1–6[acyl–]Ins–PO4–lipid. Thus, the GPI structures accumulating upon YLL031c deletion contain four mannoses and may contain HF-sensitive side chains on Man1 or Man2 but lack EtN-P on Man3 (Fig. 1).

Subcellular Localization of YLL031c—YLL031c predicts a translation product of 116 kDa. To localize the YLL031c protein, the endogenous, chromosomally encoded gene was modified by the insertion of a Myc tag at its C terminus. This YLL031c-Myc translation product (including the N-terminal signal sequence) has a predicted molecular mass of 136 kDa. The corresponding protein was detected in Western blots as a major band at about 136 kDa plus a heterogeneously glycosylated smear with another distinct band at about 300 kDa. The thus generated fragments were separated by paper chromatography, and radioactivity contained in 1-cm-wide strips was determined through scintillation counting. Standards 0–4 run on the same paper are Manα2–GlcNα–Ins with x = 0, 1, 2, 4.

The corresponding protein was detected in Western blots as a major band at about 136 kDa plus a heterogeneously glycosylated smear with another distinct band at about 300 kDa. The thus generated fragments were separated by paper chromatography, and radioactivity contained in 1-cm-wide strips was determined through scintillation counting. Standards 0–4 run on the same paper are Manα2–GlcNα–Ins with x = 0, 1, 2, 4.
the Golgi marker Och1p and is largely free of Gpi8p and Wbp1p (Fig. 7). The existence of the 300-kDa form is indicative of massive glycan elongation in the Golgi on part of YLL031c-Myc. Since only little YLL031c-Myc is found in this organelle at steady state, it may be that the protein is recirculating between Golgi and ER as has been reported for other proteins such as Emp47p, Sec12p, and Sed5p. When microsomes were incubated with proteinase K, even low concentrations of protease (10 µg/ml) readily destroyed the immunoreactivity of YLL031c-Myc (Fig. 7C). In contrast, Gpi8p, which has a large N-terminal luminal and a small C-terminal cytosolic domain of about 14 amino acids was slightly reduced in size but not destroyed by proteinase K. In view of the massive glycosylation of part of YLL031c-Myc and the presence of an N-terminal hydrophobic domain that qualifies as potential signal sequence, we interpret these findings in the sense that the N-terminal part of YLL031c containing six N-glycosylation sites is oriented luminally. The fact that proteinase K treatment of microsomes does not generate any immunoreactive low molecular weight product suggests that the C-terminal Myc tag is exposed cytosolically or that YLL031c-Myc contains a proteinase K-sensitive site close to its C terminus. The membrane topology of YLL031c thus appears to be the same as in the homologous Gpi7p (8).

**DISCUSSION**

In this study, we investigate the potential function of YLL031c in GPI anchoring. As shown in Fig. 8, YLL031c predicts a membrane glycoprotein of 1017 amino acids with an N-terminal hydrophilic domain and a C-terminal hydrophilic sequence containing numerous potential transmembrane domains, a feature that initially led to its classification as potential facilitator of membrane permeation (32).

YLL031c is homologous to Gpi7 and MCD4 of *S. cerevisiae*, but closely homologues can be found in other species such as *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans* or *Schizosaccharomyces pombe*. The Gpi7/MCD4/YLL031c gene family can be subdivided into three subfamilies of more closely related genes. MCD4, Gpi7, and YLL031c each belong to a different subfamily. All genes in this family predict proteins that have the same general structural attributes; i.e. they have an N-terminal signal sequence followed by a large hydrophilic domain and a C-terminal hydrophilic sequence containing numerous potential transmembrane domains. The hydrophilic domains of YLL031c, Gpi7, and MCD4 have a distinct homology with mammalian enzymes classified as phosphodiesterases, phosphatases, or nucleotide pyrophosphatases. This homology extends over 240 amino acids. YLL031c and *MCD4*(equivalent to *GPI7*) genes are integrated the positions of the nine potential N-glycosylation sites (vertical lines). The phosphatase motif (double bar), and two motifs conserved between the YLL031c, Gpi7, and MCD4 subfamilies (single bars) are also indicated.

**Fig. 8.** Homology of YLL031c with Gpi7 and MCD4. A, the first 395 amino acids of YLL031c are shown. Potential N-glycosylation sites and the N-terminal hydrophobic sequence for translocation of the protein into the ER are underlined. Homologies with the other *S. cerevisiae* (OSC) genes Gpi7 and MCD4 were identified using the Clustal W (version 1.8) program at EBI. This introduced a few gaps into the YLL031c sequence (dashes). In parallel, each of these three genes was aligned with its nearest neighbors in other species, namely humans, mice, *C. elegans*, and *S. pombe*. GenBankTM accession numbers and Geninfo identifiers of sequences aligned with YLL031c were T02245 GI:7513075, AAB93646 GI:2734088, and T40030 GI:7491546, aligned with Gpi7 were Q09782 GI:1175452 and T21487 GI:7500659, and aligned with MCD4 were NP_036459 GI:6912500, NP_038812 GI:7305383, and T40715 GI:7491747. These alignments reveal homologies for the YLL031c (C31) subfamily, the Gpi7 (equivalent to YKL165c) (C65), and the MCD4 (equivalent to YKL165c) (C65). Asterisks, colons, and periods indicate identity, strong similarity, and weak similarity, respectively. B, in the KYTE-Doolittle plot of YLL031c, homologies for the YLL031c (C31) subfamily, the Gpi7 (equivalent to YKL165c) (C65), and the MCD4 (equivalent to YKL165c) (C65). Asterisks, colons, and periods indicate identity, strong similarity, and weak similarity, respectively.
The six nearest pathways along the edges leading from 031a to CP2 are placed at the corners of a cube. All theoretically possible intermediates in the biosynthesis of CP2 from 031a are shown in Fig. 9. Pathways using reaction 3, leading from 031b to CP2, may also be impracticable, since YLL031c-depleted cells accumulate the substrate for reaction 3, but H2 is not observed. On the other hand, the appearance of 031b in these cells suggests that reaction 3 is possible albeit not favored. Since reactions 1 and 3 are not feasible and 3 is not favored, we may predict that the physiological pathway in normal cells proceeds mainly via the only remaining way, which is 2–3•°. Thus, the addition of ETN-P to Man1 may represent a prerequisite for the addition of further ETN-P residues. The fact that MCD4 is essential in accordance with a pivotal role of the ETN-P transfer to Man1. ETN-P is already present on M2* of gpi10–1 having the structure Mana1–6[acyl–(CH2)2–PO4–]1–4GlcN–3–6[acyl–]Ins–PO4–lipid. Also, several studies in mammalian cells document that ETN-P may be added before any other residues are attached to Man1 (11, 23, 42, 43). Nevertheless, the abundance of 031a in YLL031c-depleted cells argues that the addition of ETN-P to Man1 is not a prerequisite for the addition of mannoses (Man2, Man3, and Man4, Fig. 1).

Since YLL031c is an essential gene, we may conclude that lipid 031b cannot be used as an anchor device (i.e. that at least some essential GPI proteins cannot be attached to the ETN-P on Man1 or that this process, if it occurs, does not yield a functional GPI protein). A similar conclusion has been reached previously in that the gpi10–1 mutant does not seem to add the accumulating Mana1–6[acyl–(CH2)2–PO4–]1–4GlcN–3–6[acyl–]Ins–PO4–lipid to proteins (4).

Genes other than YLL031c may be required for the transfer of ETN-P onto Man3. In mammals this step is dependent on PIG-F, a gene encoding a very hydrophobic protein. Its exact function has not yet been elaborated (44). Further studies will be required to prove the validity of the model proposed in Fig. 9 and to show that the MCD4/GPI7/YLL031c gene family indeed encodes ETN-P-transferases.
### GPI Anchor Biosynthesis in Yeast

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