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Glycosylphosphatidylinositol Membrane Anchors in *Saccharomyces cerevisiae:* Characterization of Proteins Involved in Side Chain Modifications

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Der Leiter der Doktorarbeit

Prof. Andreas Conzelmann

Der Dekan

Prof. Alexander von Zelewsky

to my parents
and to Olivier

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Summary

In eukaryotic cells, a subset of proteins is attached to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Apart from providing stable membrane anchorage, GPI anchors are thought to be associated with other functions, such as signal transduction and protein targeting. The biosynthesis of the GPI anchor and its transfer to proteins is highly conserved in eukaryotes. The common GPI backbone, (EthN-P)-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-PO_4-lipid, is assembled by the sequential additions of sugar and ethanolaminephosphate to phosphatidylinositol. The conserved GPI core is modified by various side chains in different organisms. Side chain modification of mannoses with EthN-P has been described in mammalian cells and recently also in the yeast *Saccharomyces cerevisiae*. Several genes involved in GPI biosynthesis have been cloned by complementation of GPI-deficient yeast and mammalian mutants. We have studied GPI anchoring in *Saccharomyces cerevisiae*, because this organism is amenable to genetic studies and has advantages such as relevance to higher systems. In comparison to the complete GPI precursor glycolipid CP2, the most polar GPI accumulating in yeast *gpi7* mutants lacked a HF-sensitive side chain on Man2, suggesting that the corresponding protein, Gpi7p, might be involved in the addition of a side chain to the second mannose of the GPI core. It was therefore tempting to speculate that the proteins encoded by the two yeast homologues of *GPI7*, *YLL031c/GPI13* and *MCD4*, might play a role in the transfer of EthN-P to the first and third mannose of the GPI core. This study had as major objective the characterization of the enzymes involved in EthN-P transfer to the GPI precursor in *Saccharomyces cerevisiae*. Depletion of *GPI13* resulted in cell wall fragility, and a reduction of GPI anchor addition to proteins. The most polar GPI glycolipid that accumulated *in vivo* in cells depleted of Gpi13p lacked the bridging EthN-P on Man3 of the GPI core, suggesting that Gpi13p is involved in the transfer of EthN-P to Man3. A glycolipid with the same mobility also accumulated in *in vitro* experiments with microsomes from Gpi13p-depleted cells. Similarly, mutation or depletion of Mcd4p resulted in accumulation of GPI intermediates lacking EthN-P on Man1. These results are compatible with the idea that Gpi13p transfers EthN-P to the inner α1-2-linked mannose, and that Mcd4p transfers EtN-P onto the α1-4-linked mannose of the GPI core. A tagged form of Gpi13p was shown to be localized in the ER. In contrast, the bulk of Gpi7p was localized on the cell surface. Depletion of Gpi13p or Mcd4p induced a stress response known as the Unfolded Protein Response, providing further evidence that GPI anchoring is an essential process in *Saccharomyces cerevisiae*.

Zusammenfassung

Die Verankerung von bestimmten Proteinen an der Aussenseite der Plasmamembran mittels eines Glycosylphosphatidylinositol (GPI)-Ankers ist in eukaryontischen Zellen weit verbreitet. Es gibt Hinweise, dass GPI-Anker neben ihrer Funktion als stabiler Anker auch eine wichtige Rolle in der Uebertragung von Signalen und in der Sortierung von Proteinen spielen. Sowohl die Biosynthese des GPI-Ankers als auch seine Anheftung an Proteine sind konservierte Prozesse. Das Grundgerüst aller GPI-Anker, (EthN-P)-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-PO_4-lipid, wird durch schrittweise Addition von Zuckern und Ethanolaminphosphat an das Lipid Phosphatidylinositol, aufgebaut. Diese konservierte Grundstruktur der GPI-Anker ist in verschiedenen Organismen mit verschiedenen Seitenketten dekoriert. Dass GPI-Anker in Säugerzellen EthN-P-Seitenketten haben, ist schon seit mehreren Jahren bekannt. In der Hefe hingegen wurden EthN-P-Seitenketten erst kürzlich entdeckt. Mehrere Gene, die in der Biosynthese von GPI-Ankern eine Rolle spielen, wurden durch Komplementierung von GPI-defizienten Hefe- und Säugetierzellen kloniert. Viele zelluläre Prozesse sind sehr ähnlich in der Hefe *Saccharomyces cerevisiae* und in höheren Zellen. Weil Hefe für genetische Studien sehr geeignet ist, erforschen wir die GPI-Biosynthese in diesem Modellorganismus. Auf dem hydrophilsten GPI-Vorläufer in *gpi7* Mutanten fehlte eine Seitenkette mit Phosphodiester-Bindung auf Man2 im Vergleich zum kompletten GPI Vorläufer CP2, was vermuten liess, dass das zugehörige Enzym Gpi7p nötig ist zum Anhängen einer Seitenkette an die zweite Mannose. Daher war es verlockend zu spekulieren, dass *YLL031c/GPI13* und *MCD4*, Gene mit grosser Sequenzhomologie zu *GPI7*, eine Rolle spielen in der Anheftung von Seitenketten an die erste und dritte Mannose von GPI-Ankern. Das Ziel dieser Arbeit war die Charakterisierung von Proteinen, die für das Anheften von Seitenketten an Hefe-GPI-Anker nötig sind. Eine Verringerung der Menge des Proteins Gpi13p verursachte eine geschwächte Zellwand und eine Verminderung der Menge GPI-verankerter Proteine. Weiter führete die Verringerung von Gpi13p *in vivo* zur Akkumulierung eines GPI-Glycolipids, dem die EthN-P-Seitenkette auf der dritten Mannose fehlte, was darauf deutete, dass Gpi13p nötig ist für den Transfer von EthN-P auf Man3. Ein Glycolipid mit der gleichen Mobilität akkumulierte auch in *in vitro*-Experimenten mit Microsomen. Mutation oder Verringerung der Proteins Mcd4p verursachte die Akkumulierung von GPI-Vorläufern, denen EthN-P auf der ersten Mannose fehlte. Diese Resultate sind in Einklang mit der Idee, dass Gpi13p EthN-P auf die innere α1-2-gebundene Mannose, und Mcd4p EthN-P auf die α1-4-gebundene Mannose überträgt. Wir konnten zeigen, dass eine etikettierte Form von Gpi13p im Endoplasmatischen Reticulum (ER) lokalisiert ist. Im Gegensatz dazu war der grösste Teil des Proteins Gpi7p auf der Zelloberfläche lokalisiert. Eine Reduzierung der Menge von Gpi13p oder Mcd4p in Hefezellen führte zur Aktivierung einer Signalkaskade als Antwort auf Stress im ER, was ein weiterer Hinweis darauf ist, dass GPI-Verankerung von Proteinen für *Saccharomyces cerevisiae* ein lebenswichtiger Prozess ist.

Abbreviations

ASAM	*A. satoi* α-mannosidase
bp	base pair(s)
Cer	ceramide
CFW	Calcofluor white
CHO	chinese hamster ovary
Chx	cycloheximide
CoA	coenzyme A
COPI	coat protein complex I
COPII	coat protein complex II
CP2	complete precursor 2
CW	cell wall
d	day(s)
DAG	diacylglycerol
DFP	diisopropylfluorophosphate
DIG	detergent-insoluble glycolipid-enriched complex
DMF	dimethylformamide
Dol	dolichol
Dol-P-Man	dolichol phosphate mannose
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
EthN-P	ethanolamine phosphate
Gal	galactose
Glc	glucose
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
GPI*deAc*	GPI inositol deacylase
GPI-GnT	GPI-*N*-acetylglucosaminyltransferase
GPI-MT-I	GPI mannosyltransferase I (α1,4)

GPI-MT-II	GPI mannosyltransferase II (α1,6)
GPI-MT-III	GPI mannosyltransferase III (α1,2)
GPI-MT-IV	GPI mannosyltransferase IV (α1,2)
GPI-PLD	GPI-specific phospholipase D
GST	glutathione-*S*-transferase
h	hour(s)
HF	hydrofluoric acid
HU	high urea buffer
Ins	*myo*-inositol
IPTG	isopropyl-1-thio-β-D-galactopyranoside
JBAM	Jack bean α-mannosidase
kD	kilo Dalton
$M(IP)_2C$	mannosyl diinositoldiphosphoceramide
M1 = Man1	Man_1-GlcN-Ins headgroup or glycolipid
M2 = Man2	Man_2-GlcN-Ins headgroup or glycolipid
M3 = Man3	Man_3-GlcN-Ins headgroup or glycolipid
M4 = Man4	Man_4-GlcN-Ins headgroup or glycolipid
Man	mannose
MAP	multiple antigen peptide
MCS	multiple cloning site
min	minute(s)
MIPC	mannosyl inositolphosphoceramide
OD_{600}	optical density at 600 nm
ORF	open reading frame
P	phosphate
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI-PLC	PI-specific phospholipase C
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
PNH	paroxysmal nocturnal haemoglobinuria
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
rpm	rotations per minute

RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SGD	*Saccharomyces cerevisiae* Genome Database
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
ts	temperature sensitive
TX-100	Triton X-100
UDP-GlcNac	uridine diphosphate *N*-acetylglucosamine
UPR	unfolded protein response
UPRE	UPR element
Ura	uracil
VSG	variant surface glycoprotein
wt	wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YPD	Yeast Protein Database

1. Introduction

Discovery of GPI anchors

In virtually all eukaryotic organisms, many cell surface proteins are membrane anchored via glycosylphosphatidylinositol (GPI). Evidence for the existence of these anchors came in the 1960s with a report on the selective release of alkaline phosphatase from the surface of mammalian cells by a bacterial phospholipase C (Slein and Logan, 1963). These studies were confirmed several years later, when highly purified phosphatidylinositol-specific phospholipase C (PI-PLC) was shown to release alkaline phosphatase and other membrane proteins (reviewed by Low, 1987). In the mid-1980's, it was shown that the *Trypanosoma brucei* variant surface glycoprotein (VSG) is covalently linked to a phosphatidylinositol-containing glycolipid (Ferguson *et al.*, 1985). This finding was a major breakthrough, since VSG is present at 10^7 copies per cell, and could thus easily be purified in sufficient amount for structural analysis. The complete structure of the VSG GPI-anchor was elucidated in 1988 (Ferguson *et al.*, 1988) and its structure is shown in Figure 1-1.

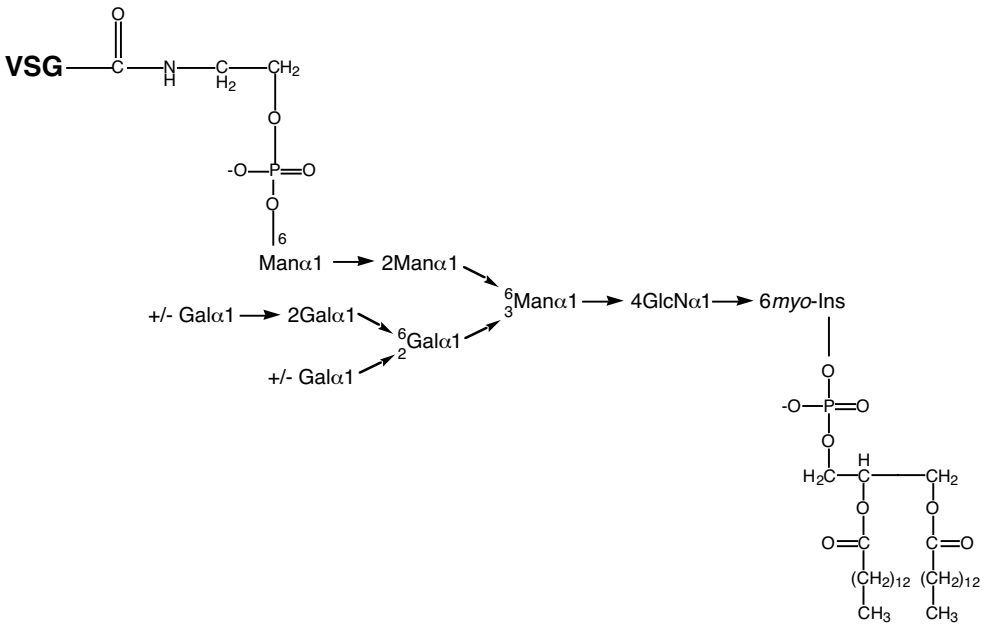

Figure 1-1. Structure of the VSG GPI anchor from *Trypanosoma brucei*.
Ins, *myo*-inositol; GlcN, glucosamine; Man, mannose; Gal, galactose.

The experimental strategies developed for the elucidation of the VSG GPI anchor structure have been used for similar studies on a number of other proteins (reviewed by McConville and Ferguson, 1993).

Occurrence and roles of GPIs

GPI-anchored proteins have been found in virtually all eukaryotes (Tiede *et al.*, 1999), and are particularly abundant in parasitic protozoa, where the majority of coat glycoproteins, surface hydrolases and receptors are GPI-anchored. In mammalian cells, GPI-anchored proteins comprise a large number of glycoproteins of various functions, including cell surface enzymes, receptors, complement defense proteins, and molecules involved in cell adhesion and activation. In *Saccharomyces cerevisiae*, GPI-anchored proteins were first reported in 1988 (Conzelmann *et al.*, 1988). Recently, GPI-anchored proteins have also been described in plants (Morita *et al.*, 1996; Takos *et al.*, 1997; Youl *et al.*, 1998).
Although the GPI glycolipids were discovered as covalently linked to cell-surface proteins, many cells contain non-protein linked or "free" GPIs (reviewed by McConville and Menon, 2000).

GPI-anchored proteins in Saccharomyces cerevisiae

In *Saccharomyces cerevisiae*, GPI-anchored proteins are found attached to the plasma membrane or as an intrinsic part of the cell wall. For some proteins, the presence of the GPI anchor has been proven, whereas for other proteins, a potential GPI anchor attachment signal has been predicted by sequence analyses, which were made possible after the sequencing of the complete *Saccharomyces cerevisiae* genome (Goffeau *et al.*, 1996). A total of 53 (Hamada *et al.*, 1998) or 58 (Caro *et al.*, 1997) GPI-anchored proteins have been predicted for *Saccharomyces cerevisiae*, and when considering also data from the YPD database (www.proteome.com/databases/index.html, July 19th, 2001), 68 proteins are predicted to be GPI-anchored. Table 1-1 shows the 52 experimentally verified GPI-anchored proteins in *Saccharomyces cerevisiae* and key references. A recent study on the number of GPI-anchored proteins however predicts 41 to 55 (depending on the level of certainty) GPI-anchored proteins in *Saccharomyces cerevisiae*, which corresponds to 0.63 % or 0.84 % of the yeast proteins (Eisenhaber *et al.*, 2001).

Table 1-1. GPI-anchored proteins in *Saccharomyces cerevisiae*.

CW and PM indicate cell wall localization and plasma membrane localization, respectively.

Gene name	Protein name	localization	Key references
YAL063c	Flo9p	CW	(Teunissen and Steensma, 1995)
YAR050w	Flo1p	CW	(Teunissen *et al.*, 1993)
YBR067c	Tip1p	CW	(Kondo *et al.*, 1991) (Van Der Vaart *et al.*, 1995) (Fujii *et al.*, 1999)
YCR089w	Fig2p	CW	(Van Der Vaart *et al.*, 1997) (Erdman *et al.*, 1998)
YCR098c	Git1p	PM	(Stratford, 1994)
YDR055w	Pst1p	CW	(Terashima *et al.*, 2000)
YDR077w	Sed1p	CW	(Van Der Vaart *et al.*, 1997) (Shimoi *et al.*, 1998)
YDR134c		CW	(Hamada *et al.*, 1999)
YDR144c	Mkc7p/Yps2p	PM	(Komano and Fuller, 1995) (Komano *et al.*, 1999)
YDR261c	Exg2p	PM	(Cid *et al.*, 1995)
YDR349c	Yps7p	PM	(Olsen *et al.*, 1999)
YDR534c	Fit1p	CW	(Hamada *et al.*, 1999)
YEL040w	Utr2p	CW	(Hamada *et al.*, 1999)
YER011w	Tir1p/Srp1p	CW	(Kowalski *et al.*, 1995)
YER150w	Spi1p	CW	(Hamada *et al.*, 1999)
YGR189c	Crh1p	CW	(Rodriguez-Pena *et al.*, 2000) (Terashima *et al.*, 2000)
YHR211w	Flo5p	CW	(Bidard *et al.*, 1994)
YIR019c	Flo11p/Muc1p	CW	(Lo and Dranginis, 1996)
YIR039c	Yps6p		(Olsen *et al.*, 1999)
YJL078c	Pry3p	CW	(Hamada *et al.*, 1999)
YJL171c		CW	(Hamada *et al.*, 1999)
YJR004c	Agα1p/Sag1p	CW	(Lipke *et al.*, 1989) (Lu *et al.*, 1994)
YJR150c	Dan1p/Ccw13p	CW	(Mrsa *et al.*, 1999)
YJR151c	Dan4p	CW	(Hamada *et al.*, 1999)
YKL096w	Cwp1p	CW	(Van Der Vaart *et al.*, 1995)
YKL097w	Cwp2p	CW	(Van Der Vaart *et al.*, 1995) (Skrzypek *et al.*, 1997) (Skrzypek *et al.*, 2000)
YKR102w	Flo10p	CW	(Teunissen and Steensma, 1995) (Guo *et al.*, 2000)
YLR040c		CW	(Hamada *et al.*, 1999)
YLR042c		CW	(Hamada *et al.*, 1999)
YLR110c	Ccw12p	CW	(Mrsa *et al.*, 1999)
YLR120c	Yps1p/Yap3p	PM	(Ash *et al.*, 1995)
YLR121c	Yps3p		(Olsen *et al.*, 1999)
YLR194c		CW	(Hamada *et al.*, 1999) (Terashima *et al.*, 2000)
YLR391w	Ccw14p/Icwp	CW	(Moukadiri *et al.*, 1997) (Mrsa *et al.*, 1999)
YMR006c	Plb2p	PM	(Fyrst *et al.*, 1999)
YMR008c	Plb1p	PM	(Lee *et al.*, 1994)
YMR215w	Gas3p	PM	(Popolo and Vai, 1999) (Hamada *et al.*, 1999)

Table 1-1 (continued). GPI-anchored proteins in *Saccharomyces cerevisiae*.

Gene name	Protein name	localization	Key references
YMR307w	Gas1p	PM	(Nuoffer *et al.*, 1991) (Vai *et al.*, 1991) (Popolo and Vai, 1999)
YNL190w		PM	(Hamada *et al.*, 1999)
YNL300w	Tos6p	CW	(Hamada *et al.*, 1999)
YNL322c	Kre1p	PM	(Roemer and Bussey, 1995)
YNL327w	Egt2p	CW	(Hamada *et al.*, 1999)
YNR044w	Aga1p	CW	(Roy *et al.*, 1991)
YOL011w	Plb3p	PM	(Merkel *et al.*, 1999)
YOL030w	Gas5p	PM	(Popolo and Vai, 1999) (Hamada *et al.*, 1999)
YOL132w	Gas4p	PM	(Popolo and Vai, 1999) (Hamada *et al.*, 1999)
YOR009w	Tir4p	CW	(Hamada *et al.*, 1999)
YOR010c	Tir2p/Srp2p	CW	(Kowalski *et al.*, 1995)
YOR214c		CW	(Hamada *et al.*, 1999)
YOR382w		CW	(Hamada *et al.*, 1999)
YOR383c		CW	(Hamada *et al.*, 1999)
YPL130w	Spo19p	CW	(Hamada *et al.*, 1999)

Yeast GPI-anchored proteins can be grouped in families with different functions. For example, the Gas1 family comprises Gas1p and its four homologues, *GAS2* to *GAS5*. Gas1p, the most abundant GPI-anchored protein in yeast, was identified as a 125 kD GPI-containing glycoprotein (Conzelmann *et al.*, 1988) and is involved in cell wall construction (reviewed by Popolo and Vai, 1999). The flocculin family comprises the proteins encoded by *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*. These proteins are involved in flocculation, i.e. the adhesion of yeast cells into clumps which sediment in the medium. The agglutinins Sag1p and Aga1p promote aggregation of cells of different mating type during mating.

Structure of GPI anchors

Shortly after the elucidation of the GPI-anchor structure of the *Trypanosoma brucei* VSG (Ferguson *et al.*, 1988), the structure of the GPI anchor of rat brain Thy-1 (Homans *et al.*, 1988) and many other proteins have been solved.

These studies have revealed a GPI core structure which is conserved in all eukaryotes, but is modified by various side chains in different organisms, in a tissue- and species-specific manner. The conserved core structure consists of ethanolaminephosphate (EthN-P) linked to the Manα1-2Manα1-6Manα1-4GlcN tetrasaccharide, which in turn is linked to *myo*-

inositol in α1-6 linkage. The carboxy-terminal amino acid of the GPI-anchored protein is covalently linked to EthN-P.

The structures of *Saccharomyces cerevisiae* GPI anchors were elucidated in 1993 (Fankhauser *et al.*, 1993). GPI anchors were prepared from crude yeast membrane protein fractions without purification of individual proteins. Figure 1-2 shows the structures of GPI anchors found in yeast.

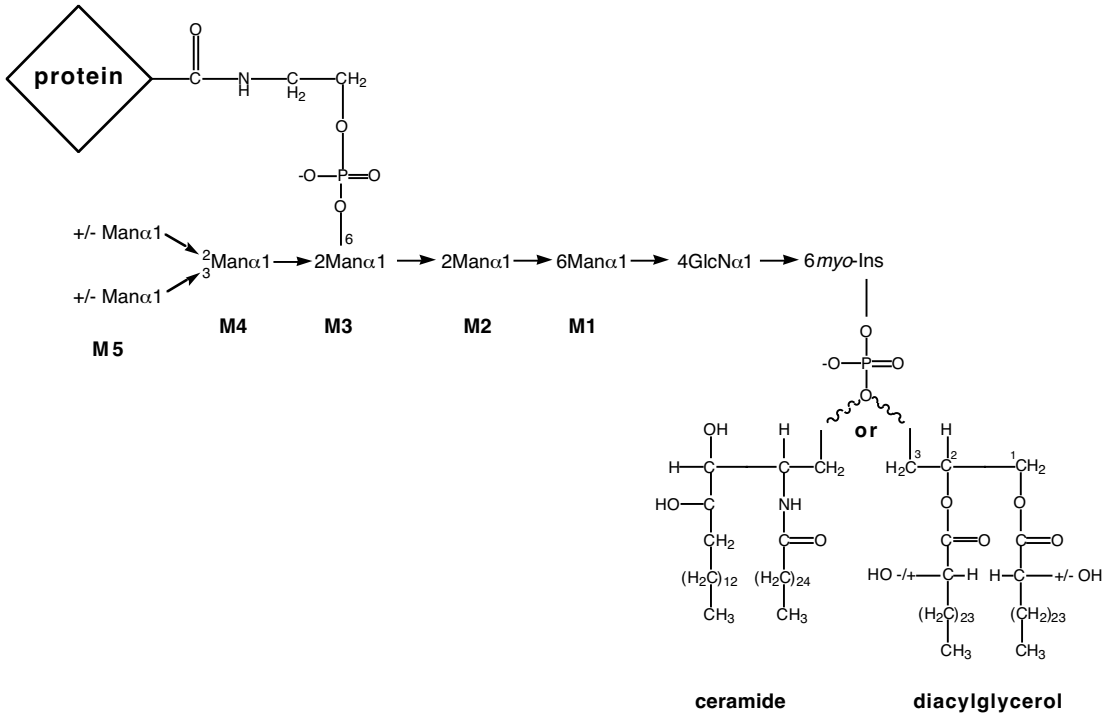

Figure 1-2. Yeast GPI anchor structures.

Ins, *myo*-inositol; GlcN, glucosamine; Man, mannose. The abbreviations M1 to M5 indicate the number of mannoses.

Biosynthesis of GPI glycolipids

Many genes encoding GPI biosynthetic enzymes as well as regulators have recently been cloned from different organisms. Characterizations of these gene products have revealed common and also different aspects of GPI biosynthesis in different organsims. GPI biosynthetic pathways have been extensively reviewed (Englund, 1993; McConville and Ferguson, 1993; Stevens, 1995; Takeda and Kinoshita, 1995; Kinoshita *et al.*, 1997; Menon *et al.*, 1997; Tiede *et al.*, 1999; Kinoshita and Inoue, 2000; McConville and Menon,

2000). The reaction steps required for GPI glycolipid biosynthesis are described below. The illustration in Figure 1-3 shows a simplified scheme of the GPI biosynthesis in *Saccharomyces cerevisiae*. The genes involved in GPI biosynthesis are listed in Table 1-2.

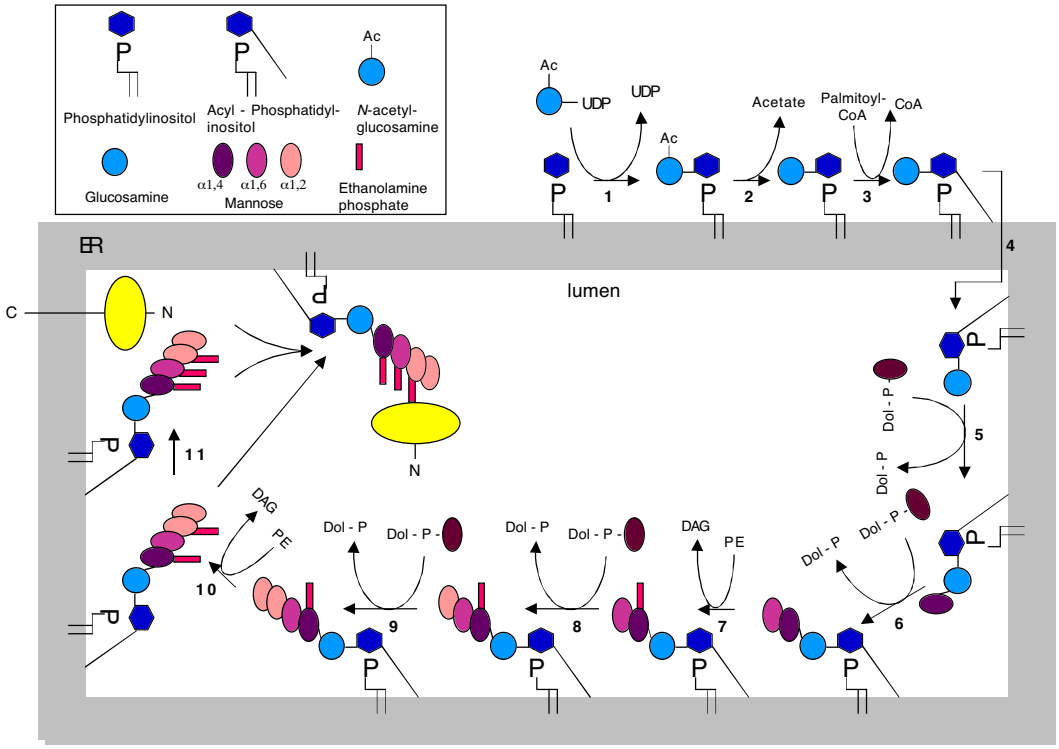

Figure 1-3. GPI biosynthesis and transfer to proteins.

A linear outline of GPI biosynthesis in *Saccharomyces cerevisiae* is depicted. However, some GPI intermediates have EthN-P modifications that suggest the existence of a branching in the GPI biosynthetic pathway (Taron *et al.*, 2000; Grimme *et al.*, 2001). The attachment of EthN-P to the α1,6-linked mannose, carried out by Gpi7p, is here shown to occur in the ER, even though only a small fraction of Gpi7p resides in the ER (Benachour *et al.*, 1999).

Synthesis of *N*-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI)

The biosynthesis of GPIs is initiated in the endoplasmic reticulum (ER) by the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) to yield GlcNAc-PI (Doering *et al.*, 1989; Masterson *et al.*, 1989). This reaction is carried out by a complex glycosyltransferase consisting of at least seven proteins in mammalian cells, PIG-A (**P**hosphatidyl**i**nositol **G**lycan A), PIG-H, PIG-C, GPI1, PIG-P, DPM2 and a 5 kD protein

not yet cloned (Watanabe *et al.*, 1998; Watanabe *et al.*, 2000)). In *Saccharomyces cerevisiae*, *GPI3/SPT14/CWH6*, *GPI2* and *GPI1* have been shown to be required for this step (Leidich *et al.*, 1995; Schönbächler *et al.*, 1995; Vossen *et al.*, 1995; Leidich and Orlean, 1996). Sequence comparisons revealed that mammalian PIG-A and *Saccharomyces cerevisiae* Gpi3p, as well as mammalian PIG-C and *Saccharomyces cerevisiae* Gpi2p are homologous proteins. Mammalian GPI1 was found as homologue of yeast Gpi1p (Tiede *et al.*, 1998; Watanabe *et al.*, 1998), *YDR437w* is homologous to *PIG-P* (Watanabe *et al.*, 2000), and *GPI15/YNL038w* has similarity to human *PIG-H* (Yan *et al.*, 2001)(data obtained on SGD). However, there is no gene homologous to DPM2 in the *Saccharomyces cerevisiae* genome. PIG-A and Gpi3p have homology to a bacterial *N*-acetylglucosaminyltransferase (GnT) and to many other glycosyltransferases, suggesting that PIG-A/Gpi13p most likely is the catalytic subunit of the GPI-*N*-acetylglucosaminyltransferase (GPI-GnT) complex (Kinoshita *et al.*, 1997). In support of this hypothesis, it was recently shown that Gpi3p (but not Gpi2p) is able to bind UDP-GlcNAc and that Gpi3p therefore probably is the catalytic subunit of GPI-GnT (Kostova *et al.*, 2000). Insights into the function of GPI1 came from recent studies in mammalian cells showing that, like in *Saccharomyces cerevisiae*, disruption of GPI1 causes only an incomplete defect in the generation of GPI-anchored proteins (Leidich and Orlean, 1996; Hong *et al.*, 1999b). It was shown that GPI1 is important for the formation of a complex comprising PIG-A, PIG-H, PIG-C and GPI1 (Hong *et al.*, 1999b).

The functions of PIG-C/Gpi2p, PIG-H and PIG-P have not yet been elucidated. The recent finding that DPM2, a regulatory subunit of the mammalian Dol-P-Man synthase, specifically binds to and positively regulates GPI-GnT, provides a first clue to understand the regulation of the GPI biosynthetic pathway at the initial step (Watanabe *et al.*, 2000). However, DPM2 is not essential for GPI-GnT activity.

De-*N*-acetylation of GlcNAc-PI

The second step of GPI biosynthesis is the generation of GlcN-PI from GlcNAc-PI (Doering *et al.*, 1989). Mammalian class J mutant cells are deficient in GlcNAc-deacetylation, and rat PIG-L as well as its yeast homologue Gpi12p are able to complement this mutation (Mohney *et al.*, 1994; Stevens *et al.*, 1996; Nakamura *et al.*, 1997; Watanabe *et al.*, 1999), suggesting that GlcNAc-PI de-*N*-acetylase is conserved between mammals and yeast. Disruption of *GPI12* in *Saccharomyces cerevisiae* is lethal, indicating that the de-*N*-acetylation step is indispensable in yeast (Watanabe *et al.*, 1999). The *PIG-L* gene

encodes a ER membrane protein, most of it residing on the cytoplasmic side. This orientation of PIG-L protein is consistent with the notion that de-*N*-acetylation occurs on the cytoplasmic side of the ER (Nakamura *et al.*, 1997).

Reversible inositol acylation

Acylation of the position 2 of the *myo*-inositol represents the third step in GPI biosynthesis in yeast and mammals (Costello and Orlean, 1992; Urakaze *et al.*, 1992), whereas in trypanosomes it occurs farther downstream in biosynthesis (Smith *et al.*, 1997). There is controversy about the precise source of the acyl group. Studies in yeast cell-free systems suggest that acyl coenzyme A (acyl-CoA) is the acyl donor (Costello and Orlean, 1992), and similar results have been obtained in CHO cells (Doerrler *et al.*, 1996). However, other studies have shown that the acyl group was transferred from endogenous membrane lipid by CoA-dependent transacylation in microsomes derived from murine lymphoma cells (Stevens and Zhang, 1994). A recent report demonstrated the coexistence of the acyl-CoA-dependent GlcN-PI acyltransferase and the CoA-dependent acyl-CoA-independent acyltransferase in rodent microsomes (Doerrler and Lehrman, 2000). The acyl group attached to the inositol ring is mainly palmitate, but this is not always the case. *In vitro* studies in *Saccharomyces cerevisiae* and the pathogenic fungus *Cryptococcus neoformans* have shown that both organisms transfer various fatty acids directly from acyl-CoAs to inositol (Franzot and Doering, 1999). The acyltransferase has not yet been cloned.

Synthesis of Dol-P-Man

Dolichol-phosphate-mannose (Dol-P-Man), the sugar donor for all three GPI core mannoses, is formed by transfer of mannose from GDP-Mannose to dolichol phosphate on the cytoplasmic side of the ER (reviewed by Abeijon, 1992). Dol-P-Man is also the mannosyl donor for *N*-glycosylation, and for protein *O*- and *C*-mannosylation (Doucey *et al.*, 1998). In *Saccharomyces cerevisiae*, the gene encoding Dol-P-Man synthase *DPM1* has been cloned and characterized (Orlean *et al.*, 1988; Orlean, 1990). Mammalian DPM1 encodes a protein with 30% identity to yeast Dpm1p, but the mammalian protein lacks a stretch of 30 C-terminal hydrophobic amino acids (Tomita *et al.*, 1998). It was then shown that mammalian Dol-P-Man synthase consists of a complex of Dpm1p and a small hydrophobic protein encoded by the DPM2 gene (Maeda *et al.*, 1998). Recently, DPM3, another subunit of the mammalian Dol-P-Man synthase was identified (Maeda *et al.*, 2000).

The observed differences in Dpm1p proteins from different organisms suggest that Dol-P-Man synthases can be divided in two classes: class 1 DPM1 proteins containing the COOH-terminal hydrophobic domain, and class 2 enzymes lacking a hydrophobic COOH-terminal domain. Class 1 proteins are found in *Saccharomyces cerevisiae*, *Ustilago maydis*, *T. brucei* (Colussi *et al.*, 1997) and *Leishmania mexicana* (Ilgoutz *et al.*, 1999b), and encode single subunit enzymes. Class 2 DPM1 proteins are found in humans, *S. pombe, Caenorhabditis* (Colussi *et al.*, 1997) and the filamentous fungus *Trichoderma reesei* (Kruszewska *et al.*, 2000), and seem to be part of a multi-component enzyme (Maeda *et al.*, 2000).

Addition of the first mannose

The first mannose is transferred from Dol-P-Man (Menon *et al.*, 1990) to the position 4 of GlcN. In yeast and mammalian cells, this mannose is transferred to GlcN-acylPI, whereas in trypanosomes, the first mannose is transferred to GlcN-PI. Recently, the human mannosyltransferase required for addition of the first mannose (GPI-MT-I) has been cloned (Maeda *et al.*, 2001). This gene, *PIG-M*, encodes a new type of mannosyltransferase containing a DXD motif characteristic of many glycosyltransferases. The finding that the lumenally oriented DXD motif is functionally important suggests that the transfer of the first mannose occur on the lumenal side of the ER. Homologues of PIG-M are found in *Saccharomyces cerevisiae* (Gpi14p (Kinoshita and Inoue, 2000)), *C. elegans* and *T. brucei* (Maeda *et al.*, 2001). Characterization of GPI-MT-I is important for two reasons. First, the orientation of the DXD active site motif allows insights into the topology of GPI biosynthesis, indicating that the flipping of the intermediate from the cytosolic to the lumenal leaflet of the ER membrane occurs after mannose addition. Second, because the substrates of GPI-MT-I are different in mammalian cells and *T. brucei*, this enzyme might be a good target for drugs that selectively inhibit GPI biosynthesis in this parasite. Some synthetic substrates able to inhibit trypanosomal GPI-MT-I have already been described (Smith *et al.*, 1999; Smith *et al.*, 2000).

Addition of the second mannose

The second mannose is transferred from the donor Dol-P-Man to position 6 of the first mannose. The $\alpha 1$,6-mannosyltransferase (GPI-MT-II) responsible for this transfer has not yet been identified. No mutants deficient in this enzyme have been reported.

The addition of ethanolamine phosphate to the first mannose

In mammalian cells, ethanolamine phosphate (EthN-P) is added to the α1,4-linked mannose residue (Man1) (Homans *et al.*, 1988; Roberts *et al.*, 1988; Puoti *et al.*, 1991; Hirose *et al.*, 1992; Kamitani *et al.*, 1992; Puoti and Conzelmann, 1993), probably at position 2 as found in the GPI anchor of Thy-1 (Homans *et al.*, 1988). In yeast, the presence of EthN-P attached to Man1 has first been revealed when analyzing the GPI intermediate that accumulated in the *gpi10-1* mutant deficient in addition of the third Man (Canivenc-Gansel *et al.*, 1998). This finding was unexpected, since previous analysis of the pool of all GPI anchors in *Saccharomyces cerevisiae* had failed to reveal EthN-P on Man1 (Fankhauser *et al.*, 1993). Recent studies in yeast have shown that phosphatidylethanolamine (PE) is the donor of the EthN-P linked to Man1 (Imhof *et al.*, 2000).

Yeast *MCD4* and its mammalian homologue *MCD4/PIG-N* are the genes involved in addition of EthN-P to Man1 (Gaynor *et al.*, 1999; Hong *et al.*, 1999a). *MCD4* was initially identified in a screen for *Saccharomyces cerevisiae* mutants deficient in bud emergence (Mondésert *et al.*, 1997). It encodes an essential ER protein containing a large N-terminal lumenal domain and multiple transmembrane domains. The temperature-sensitive *mcd4-174* mutant is defective in ER to Golgi transport of GPI-anchored proteins, secretion of proteins into the medium, morphological changes of the endomembrane system, a defect of inositol incorporation into proteins, and accumulation of multiple GPI precursor lipids (Gaynor *et al.*, 1999). Recently, *its-8*$^{+}$, the fission yeast homologue of *MCD4* has been identified (Yada *et al.*, 2001). Defects in cell wall integrity due to impaired GPI anchor synthesis in *its8-1* mutants caused morphological defects, such as abnormal cytokinesis. *its-8*$^{+}$ is not an essential gene, but disruption resulted in extremely slow growth. In mammalian cells, it has been shown that modification of the first mannose by EthN-P is not essential for attachment of GPI anchors to proteins, since the surface expression of GPI-anchored proteins was only partially affected in *Pig-n* knockout cells (Hong *et al.*, 1999a). These cells contained GPI precursors lacking EthN-P on Man1, indicating that PIG-N is involved in transfer of this side chain (Hong *et al.*, 1999a).

Yeast cells treated with the terpenoid lactone YW3548 accumulate Man_2-GlcN-acylPI (Sütterlin *et al.*, 1997b). YW3548 was isolated in a natural product screen and was initially thought to inhibit the addition of the third mannose in yeast and animal cells. However, it is now clear that YW3548 is a specific inhibitor of EthN-P addition to Man1 (Sütterlin *et al.*,

1998; Hong *et al.*, 1999a). This compound kills *Saccharomyces cerevisiae* cells at micromolar concentrations, but has no effect on GPI biosynthesis in *T. brucei* and *Plasmodium falciparum* (Sütterlin *et al.*, 1997b), where no EthN-P is added to Man1 (McConville and Ferguson, 1993). The studies on the effects of YW3548 in yeast suggest that the sequence of the GPI biosynthetic reactions in this organism are PI → GlcNAc-PI → GlcN-PI → GlcN-acylPI → Man-GlcN-acylPI → Man-Man-GlcN-acylPI → Man-(EthN-P)Man-GlcN-acylPI → Man-Man-(EthN-P)Man-GlcN-acylPI. In mammalian cells, EthN-P is most probably added at the stage of Man-GlcN-acylPI, as indicated by characterization of putative GPI precursors in mammalian cells (Hirose *et al.*, 1992).

Addition of the third mannose

The third mannose is transferred from Dol-P-Man to position 2 of the second mannose. Mammalian mutant cell lines of complementation class B have been reported to accumulate a GPI intermediate containing two mannoses (Puoti *et al.*, 1991; Sugiyama *et al.*, 1991; Puoti and Conzelmann, 1993), suggesting that GPI-MT-III is deficient. The corresponding gene was named *PIG-B* (Takahashi *et al.*, 1996). *PIG-B* encodes a protein with a large lumenal domain, suggesting that the functional site of the PIG-B protein reside on the lumenal side of the ER membrane. In yeast, *gpi10-1* mutant cells accumulate the GPI intermediate Manα1-6(EthN-P)Manα1-4GlcNα1-6(acyl)PI, suggesting that this mutant is deficient in GPI-MT-III. Interestingly, the *gpi10-1* mutant incorporated normal amounts of inositol into proteins, even though its microsomes were unable to synthesize Man_4-GPIs, suggesting that this mutant was able to make complete GPI precursors *in vivo* in spite of the biosynthetic block observed *in vitro* (Canivenc-Gansel *et al.*, 1998). It was shown that this mutant is deficient in *YGL142c/GPI10*, a yeast homologue of mammalian *PIG-B* (Canivenc-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998). Gpi10p has three homologues in the yeast genome: Alg9p and Alg12p, two non-essential proteins involved in mannosyl transfer in *N*-glycosylation (Burda *et al.*, 1996; Burda *et al.*, 1999), and Smp3p, which is required for addition of the fourth mannose in GPI biosynthesis (Grimme *et al.*, 2001).

Addition of the fourth mannose

In yeast, all GPI anchors contain a fourth, α1,2-linked mannose on the α1,2 mannose that contains the EthN-P moiety through which the GPI anchor becomes linked to protein (Fankhauser *et al.*, 1993). In mammalian cells, a portion of GPIs might also contain this

fourth mannose (Hirose *et al.*, 1992; Hong *et al.*, 2000). Consistent with this hypothesis, a fourth mannose residue has been found on 71% of rat brain Thy-1 GPI anchors, but interestingly, this extra mannose is unlikely to be present in rat thymocyte Thy-1 (Homans *et al.*, 1988), suggesting that tissue-specific side chain variation can occur.

The *Saccharomyces cerevisiae SMP3* gene encodes a protein which is required for the addition of the fourth mannose (Grimme *et al.*, 2001). *SMP3* is essential for viability, and temperature-sensitive *smp3* mutants accumulate a GPI containing three mannoses. Characterization of this GPI intermediate showed that it is a mixture of two isoforms, one with a single EthN-P on Man1, the other bearing EthN-P on Man2, providing evidence that the GPI assembly pathway in yeast may be branched. However, the most important finding was that in absence of Smp3p, addition of the bridging EthN-P to Man3 is severely, if not completely blocked (Grimme *et al.*, 2001). Smp3p-related proteins are found in *S. pombe*, *C.albicans*, *D. melanogaster* and *Homo sapiens* (Grimme *et al.*, 2001).

Addition of EthN-P to the third mannose

The addition of EthN-P to position 6 of the third mannose is an essential part of GPI biosynthesis, since it provides the "bridge" between the glycan and the protein. This EthN-P is directly transferred from phosphatidylethanolamine (PE) (Menon and Stevens, 1992; Menon *et al.*, 1993). Class F mutant lymphoma cell lines are unable to express Thy-1 at the cell surface, and accumulate multiple GPIs with one, two and three mannoses as well as side chain EthN-Ps, but the most polar GPI in these cells lacks EthN-P on Man3 (Sugiyama *et al.*, 1991; Kamitani *et al.*, 1992; Puoti and Conzelmann, 1993). Thy-1 negative class F mutants are also unable to synthesize ether lipids and make PI and GPIs that contain diacylglycerol rather than base-resistant ether lipids (Stevens and Raetz, 1990; Puoti and Conzelmann, 1993). The *PIG-F* gene restores Thy-1 surface expression and GPI anchor biosynthesis in class F mutants (Inoue *et al.*, 1993). *PIG-F* encodes a small, very hydrophobic protein. Gpi11p, a yeast protein homologous to PIG-F, has recently been characterized (Taron *et al.*, 2000). The *GPI11* gene is essential for viability and required for GPI anchoring, since yeast cells depleted of Gpi11p are unable to incorporate inositol into proteins. Depletion of Gpi11p or Pig-Fp in *Δgpi11* cells leads to accumulation of two Man_4-containing GPIs, which differ in the number and positioning of side chains. The more polar lipid is substituted on Man3, indicating that Gpi11p is not the enzyme required for the addition of EthN-P to Man3 (Taron *et al.*, 2000). Therefore, another protein is responsible for adding the bridging EthN-P. Recently, Gpi7p and Mcd4p were identified as candidate

EthN-P transferases (Benachour *et al.*, 1999; Gaynor *et al.*, 1999). *GPI7* and *MCD4* belong to a family of three yeast ORFs, *GPI7*, *MCD4* and *YLL031c*, and the finding that GPI intermediates accumulate in *YLL031c* depleted cells suggested to test *YLL031c* as a candidate EthN-P transferase (Benachour *et al.*, 1999). *YLL031c* encodes a protein of 1017 amino acids with a large *N*-terminal hydrophilic domain and multiple transmembrane domains in the *C*-terminal part. *YLL031c* as well as *MCD4* and *GPI7* contain significant homology to phosphodiesterases, further suggesting that this protein family is involved in EthN-P transfer (Benachour *et al.*, 1999; Gaynor *et al.*, 1999). *YLL031c* is an essential gene, and yeast cells depleted of *YLL031c* show a reduction of GPI anchor addition to GPI proteins as well as cell wall fragility (Flury *et al.*, 2000). In addition, they accumulate a GPI intermediate that contains four mannoses and lacks EthN-P on Man3 (Flury *et al.*, 2000; Taron *et al.*, 2000). Because of this clear implication of *YLL031c* in the GPI biosynthetic pathway, *YLL031c* was named *GPI13* (Taron *et al.*, 2000). A *C*-terminally tagged version of Gpi13p is localized in the ER (Flury *et al.*, 2000). Homologues of *GPI13* exist in *S. pombe*, *C. elegans* and *homo sapiens*, and the murine *GPI13* homologue *PIG-O* was recently identified (Hong *et al.*, 2000). *PIG-O* is not required for viability of murine embryonic carcinoma cells, but the expression of the GPI-anchored protein Thy-1 was greatly reduced, indicating that the PIG-O protein is involved in but not essential for GPI anchoring. *Pig-o* knockout cells accumulate the same major GPI intermediate as *Pig-f* mutant cells, containing three mannoses and one EthN-P on the first mannose. However, one of the two minor GPIs that accumulate in *Pig-o* disrupted cells is not present in class F cells. This intermediate most likely contains EthN-P on the third Man, and could therefore explain the residual surface expression of Thy-1 (Hong *et al.*, 2000). PIG-O and PIG-F proteins associate with each other, and PIG-F is required for the stable expression of PIG-O (Hong *et al.*, 2000).

Addition of EthN-P to the second mannose

A further EthN-P can be attached to the second mannose. This modification has been first observed in mammalian cells (Deeg *et al.*, 1992; Kamitani *et al.*, 1992; Ueda *et al.*, 1993). It has been claimed that EthN-P is added to position 6 of Man2 (Hirose *et al.*, 1992; Ueda *et al.*, 1993). This additional EthN-P seems to be attached to a GPI precursor which already contains the bridging EthN-P at Man3 (Hirose *et al.*, 1992). In *Saccharomyces cerevisiae*, *gpi7* mutants accumulate a GPI intermediate that lacks a HF-sensitive side chain on Man2, possibly EthN-P (Benachour *et al.*, 1999). *GPI7* is a non-essential gene,

and mutation or disruption of the gene affects transport and remodeling of GPI-anchored proteins, and cell wall integrity. Depletion of *GPI13* in a *Δgpi7* background results in a synthetic enhancement of the growth retardation caused by Gpi13p depletion (Flury *et al.*, 2000). *GPI7* encodes an integral membrane protein with 9-11 predicted transmembrane domains in the C-terminal part and a large, hydrophilic domain in the N-terminal part. Interestingly, the bulk of Gpi7p is localized on the plasma membrane. *GPI7* has homologues in *C. elegans* and *S. pombe* (Benachour *et al.*, 1999).

Table 1-2. Genes involved in GPI biosynthesis in yeast and mammalian cells.

Enzyme	Gene	
	S.cerevisiae	Mammals
GPI-GlcNAc transferase	*GPI3/SPT14/CWH6*	*PIG-A*
	GPI15/YNL038w	*PIG-H*
	GPI2	*PIG-C*
	GPI1	*GPI1*
	YDR437w	*PIG-P*
	§	*DPM2*
De-*N*-acetylase	*GPI12*	*PIG-L*
Acyltransferase		
Flippase		
GPI-α1,4mannosyltransferase (GPI-MT-I)	*GPI14*	*PIG-M*
GPI-α1,6mannosyltransferase (GPI-MT-II)		
Ethanolamine phosphate transferase on M1	*MCD4*	*PIG-N*
GPI-α1,2mannosyltransferase (GPI-MT-III)	*GPI10*	*PIG-B*
GPI-α1,2mannosyltransferase (GPI-MT-IV)	*SMP3*	
Ethanolamine phosphate transferase on M3	*GPI13*	*PIG-O*
	GPI11	*PIG-F*
Ethanolamine phosphate transferase on M2	*GPI7*	*hGPI7*
Transamidase	*GPI8*	*GPI8 (PIG-K)*
	GAA1	*GAA1*
	GPI16	*PIG-T*
	GPI17	*PIG-S*
Dol-P-Man synthase	*DPM1*	*DPM1*
	§	*DMP2*
	§	*DPM3*
	§	*(SL15)*

§ genes homologous to mammalian counterpart are not found in the *Saccharomyces cerevisiae* genome. For key references, see text.

Transfer of the GPI to proteins

Upon completion of its biosynthesis, the GPI is transferred to the carboxy-terminus of a pre-synthesized protein. The anchor attachment is known to occur in the ER, since GPI anchoring is still observed when vesicular traffic between the ER and the Golgi apparatus is blocked (Conzelmann *et al.*, 1988). Proteins to be GPI-anchored need to have the following features:

1) A hydrophobic N-terminal signal peptide required for entering the ER lumen.
2) A hydrophobic C-terminal GPI-attachment signal peptide which serves to transiently attach the protein in the ER membrane. During anchor addition, this signal peptide is cleaved off.
3) A suitable triplet of amino acids containing the GPI attachment site (termed "ω" site)

The transfer of the GPI to the protein occurs by a transamidation reaction, in which a putative transamidase removes the C-terminal GPI signal peptide and attaches the truncated protein to the amino group of the bridging EthN-P on the preformed GPI (Udenfriend and Kodukula, 1995). Cells deficient in transamidase activity are expected to accumulate complete GPI lipids as well GPI precursor proteins. The *Saccharomyces cerevisiae* mutants *gpi8* and *gaa1* as well as the mammalian class K mutant cell line all exhibit the transamidase deficient phenotype (Mohney *et al.*, 1994; Benghezal *et al.*, 1995; Hamburger *et al.*, 1995; Benghezal *et al.*, 1996; Chen *et al.*, 1996; Yu *et al.*, 1997). The yeast genes *GPI8* and *GAA1* were cloned and it was shown that both genes encode essential ER proteins with the bulk of the protein residing in the lumen of the ER (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996). Gpi8p belongs to a novel cysteine protease family (Benghezal *et al.*, 1996; Meyer *et al.*, 2000; Ohishi *et al.*, 2000). Homology searches have shown that *GPI8* homologues exist in man, *C. elegans*, *D. melanogaster* and *A. thaliana*, and the homology of *GPI8* with proteases suggests that Gpi8p is directly involved in the proteolytic removal of the GPI signal sequence (Meyer *et al.*, 2000). Human homologues of Gpi8p and Gaa1p have been cloned and shown to be required for GPI anchoring (Yu *et al.*, 1997; Hiroi *et al.*, 1998). Recently, homologues of Gpi8p from trypanosomes, *Leishmania* and *S. pombe* have been cloned (Hilley *et al.*, 2000; Sharma *et al.*, 2000; Shams-Eldin *et al.*, 2001). Studies with non-functional and partially functional alleles of *GPI8* suggest that Gpi8p is present in homo- or heteropolymeric complex (Meyer *et al.*, 2000). Indeed, Gaa1p and Gpi8p are found to form a complex, and Gaa1p is required for the generation of a carbonyl intermediate between the transamidase and a

precursor protein (Ohishi *et al.*, 2000). Recent *in vitro* studies provide direct evidence for an interaction between a proprotein and a transamidase subunit, and suggest that additional proteins might be part of the mammalian transamidase complex (Vidugiriene *et al.*, 2001). Indeed, two studies have identified additional subunits of the transamidase complex by affinity purification (Fraering *et al.*, 2001; Ohishi *et al.*, 2001). In the study of Fraering *et al.* (2001), digitonin-solubilized GPI transamidase complex was isolated by affinity purification from *Saccharomyces cerevisiae* membranes, and proteins contained in the complex were identified by mass spectometry. In addition to Gpi8p and Gaa1p, the complex contained Gpi16p (encoded by *YHR188c*). Gpi16p is an essential transmembrane glycoprotein. Cells depleted of Gpi16p accumulate complete GPI lipids as well GPI precursor proteins. Ohishi *et al.* (2001) identified two new components of the mammalian GPI transamidase, PIG-S and PIG-T. PIG-T is the mammalian homologue of *Saccharomyces cerevisiae* Gpi16p, and PIG-S has homology to yeast *YDR434w* (*GPI17*). *PIG-S* and *PIG-T* mouse knockout cells do not express the GPI-anchored protein Thy-1 at the cell surface and accumulate mature GPI lipids. By immunoprecipitation experiments, it was shown that PIG-T has a central role in maintenance of the transamidase complex, and Figure 1-4 shows a model based on these results.

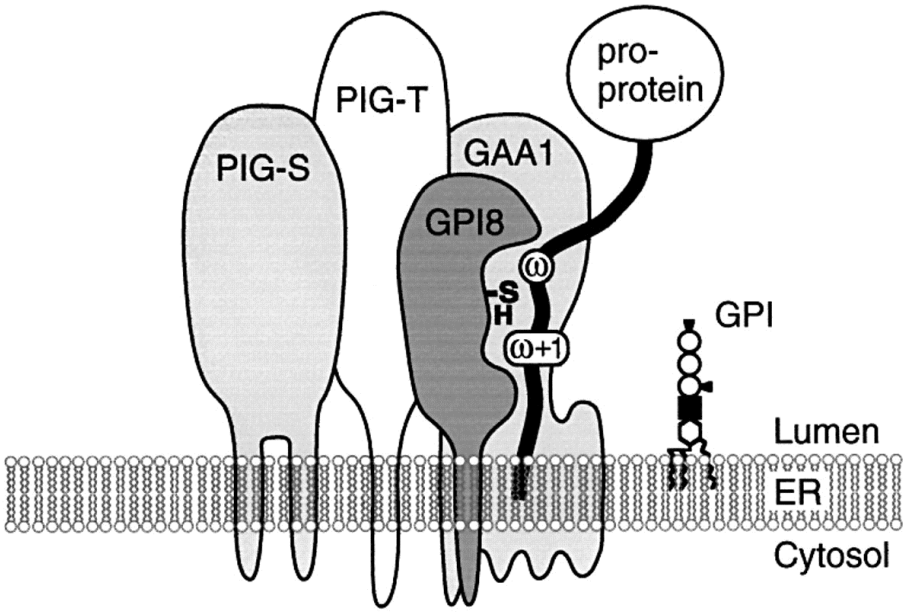

Figure 1-4. The mammalian GPI transamidase.

Model from Ohishi *et al.* (2001).

Localization and topology of GPI biosynthesis

In mammalian cells, GPI biosynthesis is initiated on the cytoplasmic leaflet of the ER (Vidugiriene and Menon, 1993). The early GPI intermediates GlcNAc-PI and GlcN-PI could be quantitatively hydrolyzed by PI-PLC in intact mammalian microsomes, indicating that they are located on the cytoplasmic leaflet of the ER (Vidugiriene and Menon, 1993), and characterization of the GlcNAc-transferase complex demonstrated that the functional transferase enzyme is cytoplasmically oriented (Watanabe *et al.*, 1996; Watanabe *et al.*, 1998; Hong *et al.*, 1999b). The topologies of subsequent biosynthetic steps are less clear. In trypanosomes and *Leishmania*, mannosylated GPI intermediates are located on the cytoplasmic side of the ER (Mensa-Wilmot *et al.*, 1994; Vidugiriene and Menon, 1994). Two interpretations of this data are possible: a) the biosynthesis of these intermediates is cytoplasmically oriented, or b) these reactions occur in the lumen of the ER, and the lipid intermediates can flip out to the cytoplasmic side. Consistent with the latter possibility, the membrane orientation of the mammalian GPI-mannosyltransferases PIG-B and PIG-M suggest that mannosylation of GPI occurs in the lumen of the ER (Takahashi *et al.*, 1996; Maeda *et al.*, 2001). This requires however a flipping step of GlcN-acylPI across the ER membrane. The transamidase components Gpi8p and Gaa1p have membrane topologies in which the bulk of the protein is lumenally oriented, supporting the idea that the mature GPI anchor is transferred to proteins in the lumen of the ER (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996).

Recent data suggests that the GPI biosynthetic pathway is spatially restricted in the ER (Ilgoutz *et al.*, 1999a; Stevens *et al.*, 1999; Vidugiriene *et al.*, 1999). Attempts to reproduce GPI biosynthesis *in vitro* using highly enriched ER preparations from thymoma cells and CHO cells were less successful than when using cell lysates (Vidugiriene *et al.*, 1999). It was shown that de-*N*-acetylation of GlcNAc-PI and subsequent reaction steps up to the singly mannosylated GPI structure containing one EthN-P side chain (intermediate H5) are largely confined to an ER subcompartment that co-fractionates with mitochondria and probably corresponds to a previously described ER fraction involved in phospholipid synthesis (Vidugiriene *et al.*, 1999). The observation that GlcNAc-PI synthase and deacylase are spatially segregated in the ER provides additional evidence for compartmentalization of GPI biosynthesis within the ER (Stevens *et al.*, 1999). In *Leishmania mexicana*, GPI biosynthetic enzymes colocalize with Dol-P-Man synthase in a stable tubular subdomain of the ER (Ilgoutz *et al.*, 1999a).

Events after attachment of the GPI to protein

Remodeling of the lipid

Studies in trypanosomes have revealed the existence of lipid remodeling, a process in which the longer fatty acid moieties of PI are sequentially removed and replaced by myristate (Masterson *et al.*, 1990). In *Saccharomyces cerevisiae*, GPI precursors contain diacylglycerol probably with C16 or C18 fatty acids, whereas the majority of protein-bound GPI anchors contain ceramide (C18:0 phytosphingosine with a C26:0 fatty acid), and a smaller fraction of proteins contain diacylglycerol with a C26:0 fatty acid (Conzelmann *et al.*, 1992; Fankhauser *et al.*, 1993; Sipos *et al.*, 1994). These findings strongly argue for a remodeling step that occurs after addition of the GPI to proteins. It was shown that at least three different remodeling pathways can lead to the observed lipid exchange: 1) remodeling from diacylglycerol to ceramide in the ER (Reggiori *et al.*, 1997); 2) remodeling from diacylglycerol to a more hydrophobic diacylglycerol probably by replacing only the acyl chains (and not by replacing the entire DAG by a different DAG) (Sipos *et al.*, 1997); and 3) remodeling to a more polar ceramide in the Golgi apparatus or on the plasma membrane (Sipos *et al.*, 1997).

Addition of the fifth mannose residue

Structural analysis of the GPI anchors found on *Saccharomyces cerevisiae* proteins has revealed the presence of a fifth mannose residue in 20% of the anchors (Fankhauser *et al.*, 1993). This side chain is linked to Man4 in α1,2 linkage or in α1,3 linkage. Whereas the fourth mannose residue is added to all yeast GPI precursors in the ER, the fifth mannose is transferred only upon arrival in the Golgi apparatus (Sipos *et al.*, 1995). Studies using secretion mutants have shown that the α1,3-mannosyltransferase is localized in the *cis*-Golgi compartment, whereas the α1,2-mannosyltransferase resides in later Golgi compartments (beyond the *sec7* block between *cis*- and *mid*-Golgi) (Sipos *et al.*, 1995). The genes that encode these mannosyltransferases have not yet been identified. So far, the presence of a fifth mannose has been described only in *Saccharomyces cerevisiae*.

Inositol deacylation

Acylation on inositol renders GPIs resistant to PI-specific phospholipase C (PI-PLC). However, most GPI-anchored proteins in mammals and other eukaryotes are sensitive to

PI-PLC, due to inositol-deacylation. This deacylation occurs in the ER, shortly after the transfer of the GPI to the protein (Chen *et al.*, 1998). In trypanosomes, the acyltransferase can be blocked by phenylmethylsulfonyl fluoride (PMSF) (Güther *et al.*, 1994), whereas the deacylase that removes the acyl chain on the inositol can be blocked by diisopropylfluorophosphate (DFP) (Güther and Ferguson, 1995), indicating that different enzymes are involved in acylation and deacylation. By exploiting the sensitivity of the trypanosome GPI inositol deacylase (GPI*deAc*) to DFP, this enzyme was affinity-labeled and purified (Güther *et al.*, 2001). The affinity-purified HA-tagged DFP-binding protein was shown to have inositol deacylase activity. However, GPI*deAc*$^{-/-}$ trypanosomes showed only reduced inositol deacylase activity, suggesting that there is another GPI*deAc* in trypanosomes. GPI*deAc* shows homology to acyloxyacyl hydrolase, a mammalian lipase. Interestingly, there are no other obvious GPI*deAc* homologues in the database, suggesting that this enzyme is unique to trypanosomes and that the other inositol deacylase(s) will be common to other organisms (Güther *et al.*, 2001).

Intracellular transport of GPI-anchored proteins

The intracellular transport of GPI-anchored proteins has recently been reviewed (Muniz and Riezman, 2000). The GPI anchor is added to newly synthesized proteins in the lumen of the ER, and GPI-anchored proteins are then transported along the secretory pathway to the cell surface. Like other secretory proteins, the ER to Golgi transport of the GPI-anchored protein Gas1p is dependent on genes specifically required for vesicular transport (Conzelmann *et al.*, 1990). Protein transport from the ER to the Golgi is mediated by COPII vesicles (reviewed in Barlowe, 1998), but COPI-coated vesicles might also me involved in forward transport from the ER to the Golgi apparatus (reviewed in Cosson and Letourneur, 1997). Components of the COPII coat assemble at the site of vesicle formation and induce the membrane to form a bud. Selective incorporation of cargo proteins into vesicles is driven by the interaction between the cargo receptors and COPII components. GPI-anchored proteins are lumenal, having the lipid part inserted in the lumenal leaflet of the ER membrane, and therefore unable to directly interact with the COPII coat on the cytosolic face of the ER membrane. p24 proteins are a large family of type I transmembrane proteins that are conserved from yeast to mammals and are found in COPI and COPII vesicles in addition to ER and Golgi membranes (Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995; Belden and Barlowe, 1996; Sohn *et al.*, 1996; Dominguez *et al.*,

1998). The exact role of the p24 family proteins is not clear, but because of their conservation through evolution, their abundance and the fact that they shuttle in transport vesicles between the ER and the Golgi compartments, these proteins might act as cargo receptors (Muniz *et al.*, 2000). *Saccharomyces cerevisiae* has eight p24 homologs, *EMP24*, *ERV25* and *ERP1* to *ERP6* (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996; Marzioch *et al.*, 1999). Deletion of *EMP24* or *ERV25* leads to a delay in the ER to Golgi transport of the GPI-anchored protein Gas1p and of the soluble, secreted protein invertase. In contrast, carboxypeptidase Y (CPY) and pro-alpha factor are secreted at wild-type rates, suggesting that Emp24p and Erv25p are candidate receptors or adaptors for Gas1p and invertase (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996), providing evidence for the existence of transmembrane adaptors that connect the lumenal GPI-anchored proteins with the cytosolic COPII coat components. Recently, it was shown that Emp24p is directly required for efficient incorporation of Gas1p into ER-derived vesicles *in vitro*, providing further evidence that Emp24p acts as a cargo receptor (Muniz *et al.*, 2000). In this study, Emp24p and Erv25p could be directly crosslinked to Gas1p in ER-derived vesicles. However, another recent study has shown that Gas1p does not depend exclusively on p24 proteins for its exit from the ER (Springer *et al.*, 2000). According to this study, the p24 proteins are not essential cargo receptors in *Saccharomyces cerevisiae*, since a strain deleted for all eight p24 proteins (p24Δ8) is viable. This result suggests that if p24 proteins are cargo receptors, transport of their cognate cargo can be achieved by different means, e.g. by using other receptors or by bulk flow.

It was recently shown that GPI-anchored proteins are segregated from other cargo proteins during ER export (Muniz *et al.*, 2001). Primary ER-derived vesicles were either immunoisolated or separated by centrifugation on sucrose gradients, and contents were analyzed. The GPI-anchored proteins Gas1p and Yps1p/Yap3p were largely excluded from vesicles that contained other secretory proteins such as the vacuolar alkaline phosphatase (ALP) and the plasma-membrane resident amino acid permease Gap1p, suggesting that GPI-anchored proteins are sorted from other proteins at the exit from the ER. This finding could explain previous observations concerning specific requirements for the transport of GPI-anchored proteins, described below.

An interesting feature of the ER to Golgi transport of GPI-anchored proteins is the requirement for Ret1p, the *alpha*-subunit of the COPI coat (Sütterlin *et al.*, 1997a). In *ret1-1* mutant cells, GPI anchoring is normal, but the ER to Golgi transport of the GPI-anchored proteins Gas1p and Yap3p/Yps1p is defective, whereas other secretory proteins are transported with wild-type kinetics. Incorporation of ceramide into proteins was normal in

ret1-1 mutant cells, but the authors have not specifically tested at which stage this lipid remodeling occurred. COPI is involved in retrieval of proteins from the Golgi apparatus to the ER (Letourneur *et al.*, 1994), and might thus be required for recycling of a protein that is required for sorting, budding or fusion of vesicles containing GPI-anchored proteins. However, since COPI vesicles can bud from the ER (Bednarek *et al.*, 1995), the requirement for COPI could also mean that GPI-anchored proteins are transported to the Golgi in COPI vesicles, whereas other secretory proteins use COPII vesicles (discussed in Muniz *et al.* (2001).

Sphingolipids are specifically required for the transport of GPI-anchored proteins from the ER to the Golgi (Horvath *et al.*, 1994; Skrzypek *et al.*, 1997; Sütterlin *et al.*, 1997a), reviewed by Dickson, (1998). When sphingolipid biosynthesis is reduced either pharmacologically or genetically, the transport of Gas1p from the ER to the Golgi is delayed. A screen for compounds that inhibit GPI anchoring or transport of GPI-anchored proteins was performed based on the different electrophoretic mobilities of the immature and the mature forms of Gas1p, and identified a fungal metabolite, myriocin (Horvath *et al.*, 1994). Myriocin inhibits serine:palmitoyl CoA transferase, the key enzyme in ceramide biosynthesis. Gas1p is GPI-anchored in yeast cells treated with myriocin, but its maturation from the 105 kD to the 125 kD form is slowed down. The same delay in Gas1p transport is also observed in *lcb1-100* mutant cells that are defective in ceramide biosynthesis (Sütterlin *et al.*, 1997a), strenghtening the importance of ceramide synthesis for transport of GPI-anchored proteins to the Golgi. The two structurally related proteins Lag1p and Lac1p/Dgt1p have recently been shown to be involved in the transport of GPI-anchored proteins, since cells disrupted for both *LAG1* and *LAC1/DGT1* show a delay in Gas1p ans Yap3p maturation (Barz and Walter, 1999). A subsequent study has shown that cells disrupted for both *LAG1* and *LAC1* lack ceramide synthase activity, suggesting that Lag1p and Lac1p either encode the catalytic subunit of ceramide synthase or are obligatory activators for the ceramide synthase in *Saccharomyces cerevisiae* (Guillas *et al.*, 2001). The observed delay in ER to Golgi transport of Gas1p and Yap3p is therefore likely to be a consequence of the defect in ceramide biosynthesis in *lag1Δlac1Δ* cells.

Lipids and proteins are organized into distinct domains within the plasma membrane. Sphingolipids and cholesterol form lateral assemblies and associate with specific proteins while excluding others (reviewed in Simons and Ikonen, 1997; Hooper, 1999; Kurzchalia and Parton, 1999; Brown and London, 2000). These domains, known as rafts, can be isolated from most mammalian cells as membrane fragments that are insoluble in non-ionic detergents (DRMs (detergent-resistant membranes); also termed DIGs (detergent-

insoluble glycolipid-enriched membranes), GEMs (glycolipid-enriched membranes), and TIFF (Triton-insoluble floating fraction)) (Brown and Rose, 1992). Multiple GPI-anchored proteins are insoluble in certain non-ionic detergents such as Triton X-100 (Hooper and Turner, 1988), and it was shown that in Madin-Darby canine kidney (MDCK) cells, the GPI-anchored protein alkaline phosphatase became detergent-insoluble only after its transport to the Golgi apparatus (Brown and Rose, 1992). This finding provided support for the formation of lipid rafts in the Golgi apparatus in mammalian cells, where sphingolipids are synthesized (Simons and Ikonen, 1997). Figure 1-5 shows a model for lipid rafts.

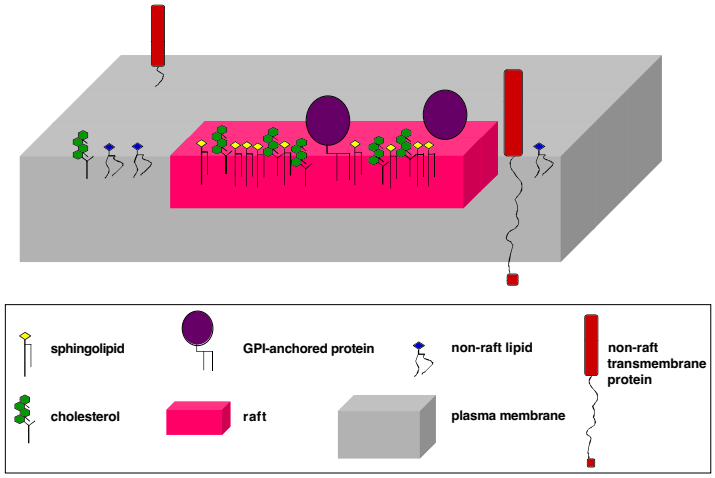

Figure 1-5. Lipid raft.

Adapted from Brown and London (2000).

A possible explanation for the ceramide requirement for the ER to Golgi transport of GPI-anchored proteins could be that ceramide microdomains form in the ER and recruit specifically GPI-anchored proteins. The physical properties of the GPI anchor could act as a signal for GPI-anchored proteins to enter the ceramide microdomains. Consistent with this idea, GPI anchor attachment was shown to be necessary for the exit of Gas1p from the ER in COPII vesicles (Doering and Schekman, 1996), and recently, detergent-insoluble glycolipid-enriched complexes (DIGs) have been detected in the yeast ER (Bagnat *et al.*, 2000). The presence of these DIGs in the ER suggests that in contrast to what has been shown for mammalian cells, lipid rafts can be formed already in the ER in yeast. Therefore, partitioning of GPI-anchored proteins into lipid rafts might be required for packaging into COPII vesicles. COPII vesicles could thus incorporate either a patch of non-raft ER membrane containing standard cargo proteins, or a raft subdomain containing GPI-

anchored proteins, allowing the production of distinct vesicles from a single COPII machinery, as shown in Figure 1-6 (discussed in Glick, 2001). In this scenario, COPI vesicles might be required for transport of sphingolipids from their site of synthesis, the Golgi apparatus, to the ER.

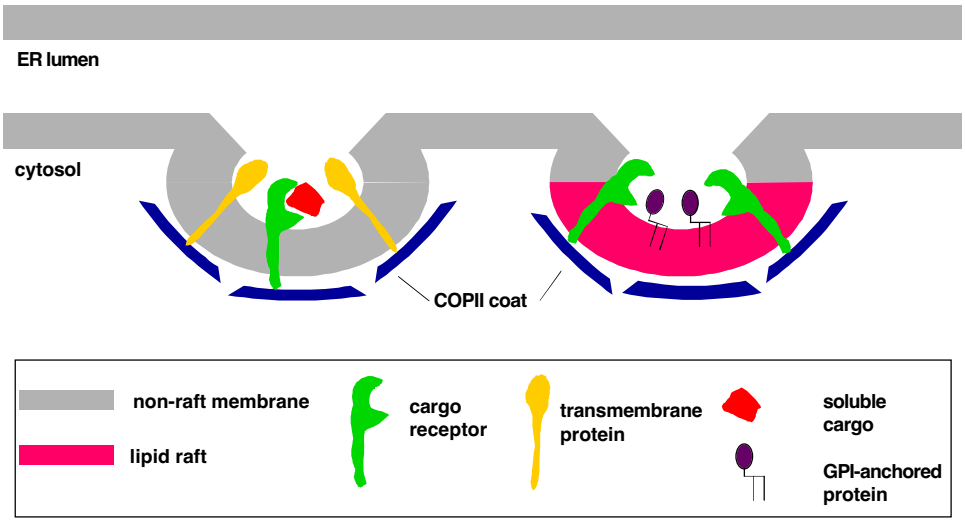

Figure 1-6. Transport of GPI-anchored proteins from the ER.
Adapted from Glick (2001).

Fate of GPI-anchored proteins

In yeast, GPI-anchored proteins are also transported to the external leaflet of the plasma membrane, but some of them are further processed and become incorporated into the cell wall (Lu *et al.*, 1994; Lu *et al.*, 1995), reviewed in Lipke and Ovalle (1998) and Kapteyn *et al.* (1999). GPI-anchored proteins that remain on the plasma membrane have a dibasic motif just N-terminal to the GPI-attachment site (ω), whereas proteins destined for the cell wall have valine, isoleucine or leucine at ω-4 or ω-5 positions, and tyrosine or asparagine at position ω-2 (Hamada *et al.*, 1999). For incorporation into the cell wall, the GPI anchor is trimmed, and lacks at least the phospholipid moietey. The glycan part of the GPI-remnant was shown to be bound to cell wall β1,6-glucans (Kapteyn *et al.*, 1996; Van Der Vaart *et*

al., 1996). Analysis of the glucan-binding sugar chain of Tip1p, a GPI-protein that gets incorporated into the cell wall, suggests that a mannose reducing end of the GPI moiety is used for binding to cell wall glucan (Fujii *et al.*, 1999), consistent with previous findings that the GPI anchor of cell wall proteins is cleaved between mannose and glucosamine (Kapteyn *et al.*, 1996; Kollar *et al.*, 1997). However, the nature of the linkage between the GPI remnant and the glucan, as well as the number of mannose residues present in the GPI remnant of Tip1p, have not been established (Fujii *et al.*, 1999). The data of Kollar *et al.* (1997) indicate that the GPI remnant consists of ethanolaminephosphate-Man_5. Another study however has found some *N*-acetylglucosamine bound to cell wall proteins, suggesting that the cleavage is between glucosamine and inositol (Montijn *et al.*, 1994). Additional studies will be required to identify the precise structure of the glucan-binding sugar chain. Figure 1-7 shows the structure of the yeast cell wall.

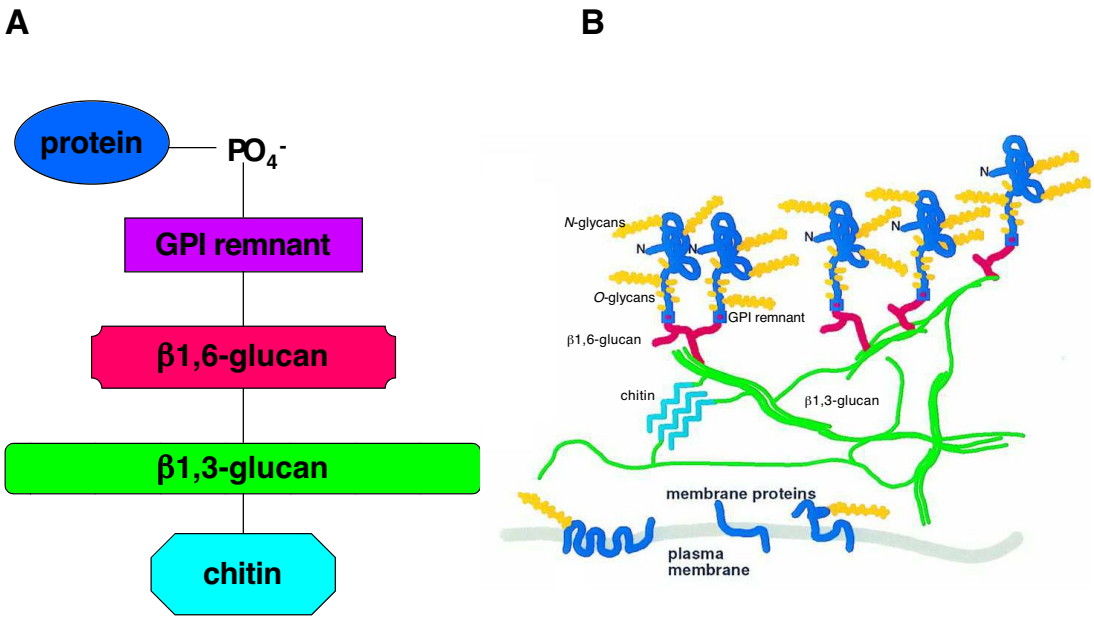

Figure 1-7. Cell wall.

A) How GPI-anchored proteins are bound to the cell wall. B) Cell wall lattice. From Lipke and Ovalle (1998).

In mammalian cells, GPI-anchored proteins attached to the cell surface can undergo internalization by endocytosis or enzymatically be released to the extracellular medium (reviewed by Nosjean, 1997). In yeast, the GPI-anchored plasma membrane protein Gce1p can be released by a endogenous (G)PI-specific phospholipase C when cells are transferred from media containing a non-fermentable carbon source to glucose medium (Müller and Bandlow, 1993). Gce1p is subsequently further processed and gets incorporated into the cell wall (Müller *et al.*, 1996). This high-molecular-weight form of Gce1p lacks inositol and ethanolamine, suggesting that this protein is linked to the cell wall differently from other GPI-anchored proteins.

Deficiencies in GPI anchoring

Saccharomyces cerevisiae mutants deficient in the GPI biosynthetic pathway are a convenient tool to identify genes involved in GPI anchoring (Leidich *et al.*, 1994; Benghezal *et al.*, 1995). GPI anchoring is essential for yeast viability, since mutations in genes required for biosynthesis of the GPI core or transfer to the protein are lethal. Restriction of GPI synthesis causes hypersensitivity to calcofluor white, a cell wall perturbing agent, and aberrant cell wall biogenesis (Vossen *et al.*, 1997).

In mammalian cells, mutation of the *PIG-A* gene causes paroxysmal nocturnal haemoglobinuria (PNH), an acquired clonal blood disease (Kinoshita *et al.*, 1997, and references therein). Intravascular hemolysis caused by the patients own complement is a major clinical symptom of PNH. Somatic mutation of *PIG-A* in a hematopoietic stem cell leads to a lack of GPI-anchored proteins. *PIG-A* is an X-linked gene (Takeda *et al.*, 1993), and since there is only a single functional X-chromosome in both male and female somatic cells, mutations affecting GPI biosynthesis are more likely found in *PIG-A* than in any autosomal GPI biosynthetic gene. In mice, targeted disruption of *PIG-A* is lethal during embryonic development, indicating that the total absence of GPI-anchored proteins causes a lethal effect (Kawagoe *et al.*, 1996).

In parasitic protozoa, GPI-anchored glycoproteins and glycosylated PIs are abundant cell surface constituents (reviewed by Tiede *et al.*, 1999). In humans, such parasites can cause important diseases, such as sleeping sickness and malaria. GPI-anchored cell surface molecules play a major role in parasite infectivity and survival, and therefore the inhibition of GPI pathways might attenuate pathogenicity and viability of these organisms. It is hoped that differences in properties of GPI biosynthetic enzymes in different species can be

exploited for drug design. Indeed, a GlcNAc-PI analogue containing 2-*O*-octyl-D-*myo*-inositol or the GlcNAc-PI diastereoisomer containing L-*myo*-inositol (GlcNAc-P(L)I) are substrates for the trypanosomal De-*N*-acetylase, but not for the HeLa enzyme, suggesting that selective inhibition of the trypanosomal de-*N*-acetylase may be possible and that this enzyme should therefore be considered as a possible therapeutic target (Sharma *et al.*, 1999).

Aim of the Thesis Work

Side chain modification of GPI anchors with EthN-P has been described for several years in mammalian cells, but not in parasites. In *Saccharomyces cerevisiae*, similar side chain modifications were only recently found to occur (Canivenc-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998; Benachour *et al.*, 1999). In 1998, at the beginning of this thesis, there were no reports on the genes involved in the transfer of EthN-P in the literature. In our laboratory, studies with the *gpi7* mutant have shown that the corresponding protein, Gpi7p, might be involved in the addition of a HF-sensitive side-chain to Man2 of the GPI core, since this mutant lacked a HF-sensitive side chain on Man2 in comparison to the complete precursor glycolipid CP2. It was tempting to speculate that the proteins encoded by *GPI7* and its two yeast homologues, *YLL031c* and *MCD4*, might play a role in the transfer of EthN-P to the GPI core.

The work reported in this thesis is a characterization of the enzymes involved in EthN-P transfer to the GPI precursor in *Saccharomyces cerevisiae*.

2. Role of *GPI13/YLL031c* in GPI biosynthesis

Summary

GPI anchors serve to attach certain secretory proteins to the outer leaflet of the plasma membrane. The biosynthesis of the GPI anchor and its transfer to proteins is highly conserved in eukaryotes. In the yeast *Saccharomyces cerevisiae*, the genes *GPI7* and *MCD4* have been shown to be involved in GPI anchor biosynthesis. This thesis work had as main objective the characterization of *YLL031c/GPI13*, a yeast open reading frame predicting a further homologue of *GPI7* and *MCD4*. During this work, we have shown that *YLL031c/GPI13* encodes an essential membrane protein that is involved in GPI anchoring. Cells depleted of Yll031c-protein (Gpi13p) accumulated two GPI intermediates, both lacking EthN-P on Man3, suggesting that Gpi13p transfers the bridging EthN-P onto the GPI core. Consistent with a role for Gpi13p in GPI anchoring, a reduction of GPI anchor addition to proteins and cell wall fragility was observed in yeast cells depleted of Gpi13p. A tagged form of Gpi13p was functional and shown to be localized in the ER.

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

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

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***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 Manα1–2[NH_2-$(CH_2)_2$-PO_4→]Manα1–2Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]inositol-PO_4-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-depleted cells accumulate GPI intermediates with the structures Manα1–2Manα1–2Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]inositol-PO_4-lipid and Manα1–2Manα1–2Manα1–6Manα1–4GlcNα1–6[acyl→]inositol-PO_4-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 mannoses of the GPI anchor, respectively.**

The structural analysis of GPI[1] anchors revealed that different organisms add different side chains to the universally conserved Manα1–2[NH_2-$(CH_2)_2$-PO_4→]Manα1–2Manα1–6Manα1–4GlcNα1–6inositol-PO_4-lipid core structure (Fig. 1) (1). In particular, human and all other mammalian cells invariably add an EtN-P group onto Man1 (Fig. 1), and in a few cases they also add an EtN-P group to Man2 (for a review, see Ref. 2). Although no EtN-P side chains had been found in a study of a pool of protein-derived GPI anchors of *Saccharomyces cerevisiae* (3), it recently was shown that the GPI intermediate M2* accumulating in a *gpi10* mutant has the structure Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]inositol-PO_4-lipid (4). Moreover, the complete GPI anchor precursor lipid CP2 that can be observed in mutants such as *pmi40* or in transamidase mutants (5–7) contains phosphodiester-linked side chains on both Man1 and Man2 (4, 8). This suggests that the side chains on Man1 and Man2 may have been invented before the separation of the lineages leading to present day yeast and mammals. Recent studies have implicated *MCD4* and *GPI7* in the transfer of EtN-P groups onto GPI structures in yeast. *MCD4* is essential, and *mcd4–174* cells show an almost complete defect in GPI anchor addition to proteins at the restrictive temperature (9, 10). Deletion of PIG-N, a mammalian homologue of *MCD4*, leads to a reduced surface expression of GPI proteins and loss of an enzyme activity that adds EtN-P to Man1 (11). *GPI7* is not essential, but its deletion slows the GPI anchor addition to newly synthesized proteins, produces cell wall fragility, and blocks biosynthesis of GPI lipids at a premature stage. Of the several abnormal GPI lipids accumulating in Δ*gpi7* cells, even the most complete one lacks the phosphodiester-linked substituent on Man2, a finding that relates *GPI7* to the addition of a side chain to Man2 (8). The idea that *MCD4*, *GPI7*, and their homologue YLL031c may all be involved in the transfer of EtN-P groups has been fostered by their pronounced homology with bacterial, viral, plant, and mammalian phosphodiesterases, phosphatases, and pyrophosphatases (8, 10).

Here we investigate whether YLL031c plays any role in GPI biosynthesis and, specifically, in the addition of EtN-P- groups to GPI structures.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials—S. cerevisiae strains were FBY413 *(MATa ura3–52 leu2-Δ1 trp1-Δ63 his3-Δ200)*, FBY1102 *(MATa ura3–52 leu2-Δ1 trp1-Δ63 his3-Δ200 yll031c::HIS3-pGAL1, 10-YLL031c)*, FCEN010a *(MATa ura3–52 trp1-Δ63 his3-Δ200 YNR019w::KanMX4)*, FBY1103 *(MATa ura3–52 trp1-Δ63 his3-Δ200 yll031c::HIS3-pGAL1,10-YLL031c YNR019w::KanMX4)*, FBY1104 *(MATa ura3–52 trp1-Δ63 his3-Δ200 mcd4::HIS3-pGAL1,10-MCD4 YNR019w::KanMX4)*, FBY182 *(MATα ade2–1 ura3–1 leu2–3,112 his3–11,15 gpi7::KanMX4)*, FBY1106 *(MATa yll031c::HIS3-pGAL1, 10-YLL031c gpi7::KanMX4 leu2 ura3 his3)*, FY1679 *(MATa/α ura3–52/ura3–52 leu2-Δ1/+ trp1-Δ63/+ his3-Δ200/+)*, FBY1105 *(MATa/α ura3–52/ura3–52 leu2-Δ1/+ trp1-Δ63/+ his3-Δ200/+ YLL031c::KanMX4/YLL031c)*, FBY1107 *(MATa ura3–52 leu2-Δ1 trp1-Δ63 his3-Δ200 yll031c::YLL031c-13Myc-KanMX6)*, FBY122 *(MATa ade2–1 ura3–1 leu2–3,112 his3–11,15 trp1–1 gpi8–1 gpi7–1)*, FBY169 *(MATα ade2–1 can1–100 ura3–1 leu2–3,112 his3–11,15 trp1–1 gpi10–1)*, and HMSF176 *(MATa sec18–1)*. Maintenance and growth conditions have been described (7). CFW-containing plates were made as described (12), with the exception that a 100 mg/ml filter-sterilized CFW stock solution was used.

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[1] 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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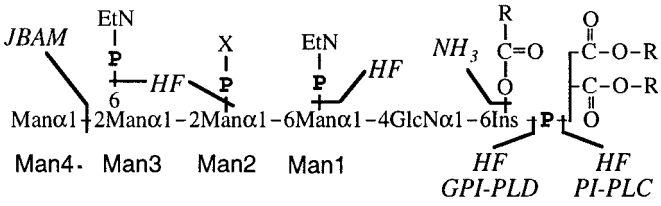

FIG. 1. **Presumed structure of the complete yeast precursor glycolipid CP2.** Relevant cleavage procedures are indicated. Man1, Man2, Man3, and Man4 are used here to designate the α1–4-linked, α1–6-linked, and α1–2-linked mannoses, respectively. *X* indicates an HF-sensitive group that is not yet defined chemically. *R*, alkyl; *P*, phosphate.

Materials were obtained from the sources described recently (4). Oligonucleotides were from Microsynth GmBH (Balgach, Switzerland), Quantazyme ylg from Quantum Biotechnologies Inc. (Montreal, Canada), Calcofluor White (CFW) from Sigma, and antibodies to carboxypeptidase Y from Molecular Probes, Inc. (Eugene, OR). Rabbit antibodies to Yap3p and Cwp1p were kindly donated by Dr. Y. Bourbonnais (Université Laval, Québec, Canada) and Dr. H. Shimoi (National Institute of Brewing, Higashihiroshima, Japan), respectively. Rabbit antibodies to Och1p and Wbp1p were gifts from Dr. Y. Jigami (National Institute of Bioscience and Human Technology, Ibarasaki, Japan) and Dr. M. Aebi (Mikrobiologisches Institut, ETH Zürich, Switzerland), respectively. A mouse monoclonal anti-Myc antibody, clone 9E10, was obtained from Babco (Richmond, CA), and goat anti-mouse IgG-peroxidase conjugate was from Sigma.

Conditional Expression of YLL031c and MCD4—Conditional expression of YLL031c was achieved by the insertion of the *GAL1,10* promoter in front of the chromosomal YLL031c gene as described (13). Briefly, the *HIS3* marker flanked by the *GAL1,10* promoter was PCR-amplified using pTL26 as template and the following two adapter primers: 031GalFor (5′-AAGATCAAAAAAGGAATAGAAGCATATGTTTTAAGGGCAACGCCGctcttggcctcctctag-3′) with 17 nt of homology to the pTL26 vector (lowercase) and 45 nt of homology to a 5′-flanking sequence of YLL031c (uppercase) and oligonucleotide 031GalRev (5′-AAGAATCGACTTTTTAATTGTCTTTTCATC**CAT**ATTACGGGAGCTcgaattccttgaattttcaaa-3′) with 45 nt of homology to the 5′-end of YLL031c ending 12 nt upstream of the start codon (boldface type) and 21 nt of homology to the pTL26 vector (lowercase type). This PCR-generated DNA fragment was used to transform the strains FCEN010a and FBY413, yielding FBY1103 and FBY1102, respectively. Correct targeting of the inserted promoter was verified by whole yeast cell PCR (14), using primers A3Gal (5′-gagcagttaagcgtattactg-3′) and 031A4 (5′-catcaatgaaagtcggtaagg-3′) yielding a 0.7-kilobase pair DNA fragment.

The genomic *MCD4* was placed under the control of the *GAL1,10* promoter by the same strategy as described above for YLL031c, using the following two adapter primers for PCR amplification from the pTL26 as the template: 165GalFor (5′-GAACCGTTCTTTACTATATATTCAACAACCCATCTTCGACCAAAGctcttggcctcctctag-3′) with 17 nt of homology to the pTL26 vector (lowercase type) and 45 nt of homology to the *MCD4* flanking sequence (uppercase type) and oligonucleotide 165GalRev (5′-GACACCAACAGCCAGAAGCGTCGTTCTGGTTTTGTTCCA**CAT**TTTcgaattccttgaattttcaaa-3′) with 45 nt of homology to *MCD4* ending 3 nt upstream of the start codon (boldface type) and 21 nt of homology to the pTL26 vector (lowercase type). This PCR-generated DNA fragment was used to transform the strain FCEN010a. Correct targeting of the *HIS3*-marked *GAL1,10* promoter was verified by whole yeast cell PCR using primers A3Gal (5′-gagcagttaagcgtattactg-3′) and 165A4 (5′-agcaatcatagcaacatgacc-3′) yielding a 0.5-kilobase pair DNA fragment.

Disruption of YLL031c—For deletion of YLL031c, a short flanking homology (SFH) replacement cassette was synthesized by PCR amplification of the KanMX4 module of pFA6a-KanMX4 (15) with the following primers: primer 031-S1 (5′-GATGAAAAAATAATATACAAATCGCGAATAAAGAAATTTCAAcgtacgctgcaggtcgac-3′) with 42-nt homology to amino acids 16–29 of YLL031c and 18-nt homology to the KanMX4 module (lowercase type) and primer 031-S2 (5′- TATAGTATATTTGTAAGTAAAGAGTGGAAATGAAGTTCG**TCA**TTatcgatgaattcgagctcg-3′) with 19 nt of homology to the KanMX4 module (lowercase type) and 44 nt of homology to a sequence near the 3′ end of YLL031c (uppercase type) comprising the stop codon (boldface type). Since gene replacement with this SFH cassette proved to be difficult, the SFH cassette was used as a template to construct a long flanking homology cassette. This long flanking homology replacement cassette was synthesized as described (16). In a first step, the primer pair K1 (5′-CATGGTACAATTGCAAAGT-3′) and L2 (5′-TTGAAATTTCTTTATTCGCGATTTGTATATTAT-3′) as well as the primer pair L3 (5′- AA**TGA**CGAACTTCATTTCCACTCTTTACTTACAAATATAC-3′) and A4 (5′-GCTGCAGAAAAGAGATGC-3′) were used to amplify two fragments of genomic DNA corresponding to the promoter region including the first 29 codons of the open reading frame and the terminator region, respectively. The 5′-parts of primers L2 and L3 were designed to anneal, respectively, to the 5′- and 3′-ends of the SFH replacement cassette, which are homologous to YLL031c. In the second step, a PCR was performed using the SFH cassette as template and the two PCR-generated fragments from the first step as primers, yielding a replacement cassette with long flanking regions having homology to the YLL031c gene. The long flanking homology cassette was used to transform the diploid WT strain FY1679, homozygous for YLL031c. The correct targeting of the KanMX4 replacement cassette to the YLL031c locus was verified by whole yeast cell PCR using primer K1, primer 031-A5 (5′-catcaatgaaagtcggtaagg-3′) annealing inside the YLL031c coding sequence, and the K2 primer of the KanMX4 module (5′-gtcgcacctgattgcccg-3′), yielding a 900-bp DNA fragment for the disrupted gene and a 748-bp DNA fragment for the WT YLL031c gene.

Tagging of YLL031c—A Myc tag was inserted at the C terminus of the genomic copy of YLL031c by homologous recombination with an insertion cassette containing the Myc tag and the selectable KanMX6 marker as described (17). The insertion cassette was obtained by using plasmid pFA6a-13Myc-kanMX6 (17) as template and the oligonucleotides F2 and R1 as target gene-specific primers (F2, 5′-AGTGGGAGATTGATTAAGCACATAAATGACATTTTTTGG**AAA**cggatccccgggttaattaa-3′, with 20-nt homology to the pFA6a-13Myc-kanMX6 sequence (lowercase type) and 42-nt homology to the sequence of YLL031c immediately upstream of the stop codon (uppercase type), including the codon of the last amino acid (boldface type); R1, 5′-ATATATAGTATATTTTGTAAGTAAAGAGTGGAAATGAAGTTCGgaattcgagctcgtttaaac-3′ with 42-nt homology to the genomic sequence of YLL031c immediately downstream of the stop codon (uppercase) and 20-nt homology to the pFA6a-13Myc-kanMX6 plasmid sequence). This PCR fragment was used to transform the WT strain FBY413, yielding FBY1107. The correct targeting of the PCR-made module was verified by whole yeast cell PCR using the primer K2 of the kanMX module and oligonucleotide C1-Myc (5′-CTGACACTGTGGTCACAGCC-3′), producing a 1577-nt fragment.

Cellular Localization and Protease Sensitivity of YLL031c-Myc Protein—The subcellular localization of YLL031c-Myc was determined essentially as described (18). Briefly, 100 optical density units of exponentially growing FBY1107 cells were washed with 10 mM NaN_3 and converted to spheroplasts by incubation for 1 h at 30 °C with Quantazyme in 1.4 M sorbitol, 10 mM NaN_3, 50 mM K_2HPO_4, pH 7.5, 40 mM 2-mercaptoethanol. Spheroplasts were resuspended in lysis buffer (20 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 250 mM sorbitol, 1 mM magnesium acetate, 20 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml antipain, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin) and homogenized by 20 strokes in a Dounce homogenizer. The crude lysate was centrifuged twice at 1000 $\times$ *g* for 5 min to remove unlysed spheroplasts. The cleared supernatant was then centrifuged at 13,000 $\times$ *g* for 15 min to generate pellet P13 and supernatant S13. S13 was centrifuged at 100,000 $\times$ *g* for 60 min in a Sorvall AH-650 swing out rotor to generate pellet P100 and supernatant S100. Pellet fractions were dissolved and denatured in high urea buffer (8 M urea, 5% SDS, 200 mM Tris-HCl, pH 6.8, 20 mM EDTA, bromphenol blue, 15 mg/ml dithiothreitol) by sonication and incubation at 95 °C for 5 min. The S100 fraction was trichloroacetic acid-precipitated before denaturation in high urea buffer.

For probing the surface exposure of YLL031c-Myc, exponentially growing FBY1107 cells were converted to spheroplasts as described above but using zymolyase-20T. Spheroplasts were either mock-treated or treated with proteinase K in the presence or absence of 0.5% Triton X-100 for 30 min on ice. Phenylmethylsulfonyl fluoride was then added to a final concentration of 4 mM, and samples were kept on ice for 15 min. Finally, spheroplasts were placed on a cushion of 1.5 M sorbitol, 50 mM K_2HPO_4, pH 7.5, 20 mM EDTA, 10 mM NaN_3, 10 mM NaF and were centrifuged. YLL031c protease sensitivity in microsomes was examined by protease K digestion essentially as described (8), except that the membrane pellet was resuspended in 600 μl of lysis buffer before protease digestions. All samples were denatured during 5 min at 95 °C in reducing sample buffer containing 20 mM EDTA and analyzed by SDS-polyacrylamide gel electrophoresis in 6–10% gels (19) followed by Western blotting.

Miscellaneous Methods—Western blots were revealed using the chemiluminescence ECL kit from Amersham Pharmacia Biotech.

Previously described procedures were used to label cells with [2-^{3}H]Ins and for lipid extraction (20).

In vitro biosynthesis of GPIs with microsomes was performed as described previously (4), with the exception that spheroplasts were sometimes prepared using Quantazyme ylg instead of zymolyase-20T. Desalted lipid extracts were analyzed by ascending TLC using solvent 1 ($CHCl_3$/CH_3OH/H_2O, 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 radioscanning (LB 2842; Berthold AG, Regensdorf, Switzerland). Thereafter, TLC plates were sprayed with EN^{3}HANCE and exposed to film (X-Omat; Eastman Kodak Co.) at −80 °C.

Soluble head groups were obtained from purified radiolabeled glycolipids through limiting methanolic NH_3 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. satoi* 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/H_2O (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 KanMX4 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, thus 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 Δ*gpi7* background, the doubling time of YLL031c-depleted cells, after 24 h on glucose, had risen to 7.7 h, while Δ*gpi7* 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 Δ*gpi7* 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 Δ*gpi7* background. Other GPI proteins such as Yap3p and Cwp1p are also affected. Although the details of the

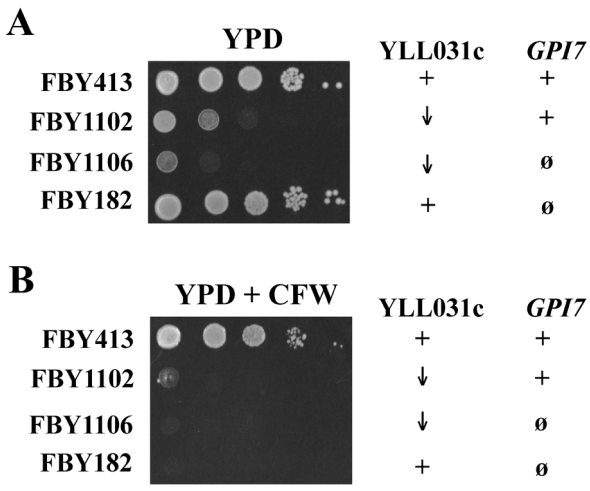

FIG. 2. **Characterization of YLL031c.** From cell suspensions containing 2.5 × 10^7 cells/ml, we prepared sequential 1:10 dilutions. 3-μl aliquots of the various dilutions were spotted on agar plates containing either YPD medium (*A*) or YPD with 1.5 mg/ml of CFW (*B*). Plates were photographed after 3 days at 30 °C. The amounts of YLL031c protein and Gpi7p in the various strains are indicated on the *right*. +, normal or elevated due to induction of *GAL1,10* promoter; ↓, depleted due to repression of *GAL1,10* promoter; ø = non existent due to deletion of the gene.

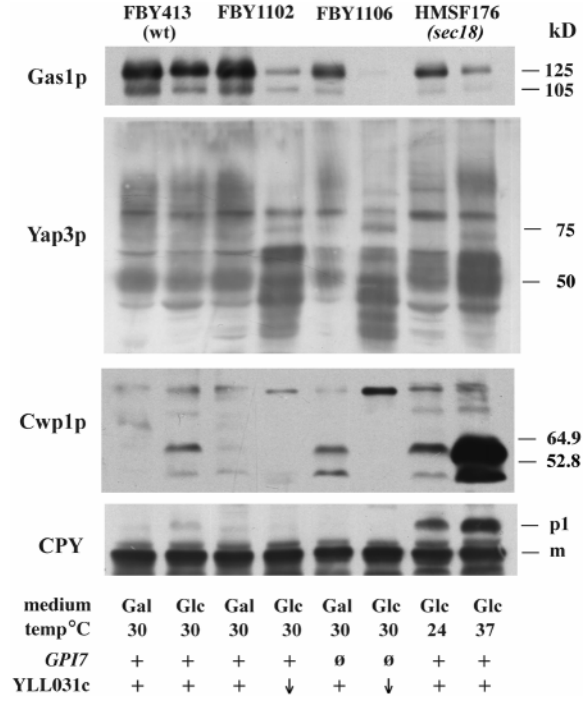

FIG. 3. **Depletion of YLL031c leads to a depletion of mature and accumulation or depletion of immature forms of GPI proteins.** Cells indicated at the *top* were grown for at least four generations at 30 °C on either Gal or Glc except for *sec18* cells, which were grown at 24 °C but were shifted to 37 °C 2 h before extraction. Exponentially growing cells were lysed by boiling for 5 min in reducing sample buffer and processed for SDS-polyacrylamide gel electrophoresis on 7.5 or 10% gels (19) followed by Western blotting with antibodies against Gas1p, Yap3p, Cwp1p, or carboxypeptidase Y (*CPY*). The expected amounts of Gpi7p and YLL031c protein in these cells are indicated as in Fig. 2.

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 form 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 *myo*-[^{3}H]inositol ([^{3}H]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 *gpi10–1*, which has the structure Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]Ins-PO_4-lipid (Fig. 4*B*, *lanes 3* and *4*) (4). The more polar lipid 031b has a lower R_F than M2* but a higher R_F than M4, the GPI lipid that accumulates in Δ*gpi7* cells and has the structure Manα1–2[NH_2-$(CH_2)_2$-PO_4→]Manα1–2-Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]Ins-PO_4-lipid (Fig. 4*A*, *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. 4*C*, *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 R_F than the M4 made by Δ*gpi7* microsomes (Fig. 4*C*, *lanes 3*, *4*, *10*, and *11*). This *in vitro* generated lipid comigrates with *in vivo* made 031b (Fig. 4*C*, *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 Δ*gpi7* 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. 4*C*, *lanes 4*, *6*, and *11*). The severely affected *mcd4–174* mutant has been reported to accumulate only trace amounts of abnormal [^{3}H]Ins-labeled lipids (10). We find the same if Mcd4p is depleted using the repressible *GAL1,10* promoter (Fig. 4*A*, *lane 5*).

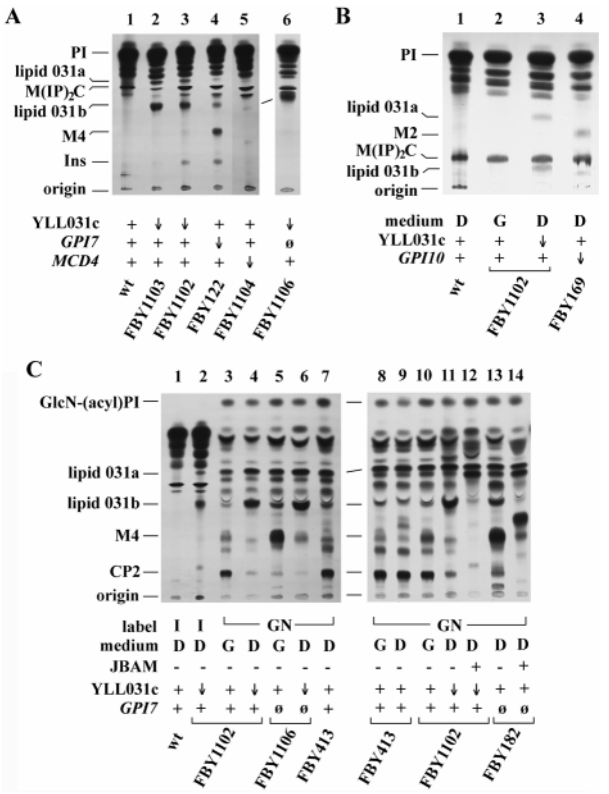

FIG. 4. **YLL031c-depleted cells accumulate at least two abnormal GPI lipids.** *A* and *B*, exponentially growing cells were radiolabeled with [^{3}H]Ins at 37 °C (4 μCi/1–2 × 10^7 cells), and desalted lipid extracts were analyzed by TLC using solvent 1 (*A*) or solvent 2 (*B*) and fluorography. *C*, for *lanes 1* and *2*, cells were labeled with [^{3}H]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-[^{3}H]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*). *M(IP)$_2$C*, mannosyldiinositolphosphorylceramide. The amounts of YLL031c activity and Gpi7p, Mcd4p, and gpi10 activity are indicated as in Fig. 2.

Structural Characterization of Lipids 031a and 031b—YLL031c-depleted cells were labeled with [^{3}H]Ins, and lipids 031a and 031b were purified by preparative TLC. Their structural analysis 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 Man_4-GlcN-Ins core structure (Fig. 5*A*), that two or three of its mannoses can be removed by JBAM (Fig. 5*C*), whereas the same enzyme removes all four mannoses from the HF dephosphorylated head group (Fig. 5*D*). If the HF-treated core structure is treated with *Aspergillus satoi* α-mannosidase, a linkage-specific exo-α-mannosidase cleaving α1–2 bonds, one obtains Man_2-GlcN-Ins (Fig. 5*B*). These data strongly suggest that the bulk of lipid 031b has the structure Manα1–2Manα1–2Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]Ins-PO_4-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. 4*C*, *lanes 11* and *12*) is in agreement with the

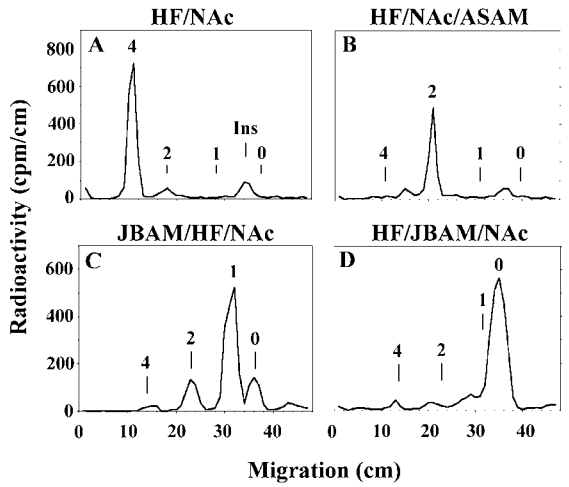

FIG. 5. **Analysis of the head group of lipid 031b using HF, JBAM, and *A. satoi* α-mannosidase (*ASAM*).** FBY1102 was labeled with [^{3}H]Ins at 30 °C, and lipid 031b was purified by two rounds of preparative TLC and used to prepare head groups. Head groups were subjected to the following sequential treatments. *A*, HF followed by *N*-acetylation; *B*, HF followed by *N*-acetylation, desalting, and then *A. satoi* α-mannosidase; *C*, JBAM followed by HF and then N-acetylation; *D*, HF followed by desalting, then JBAM, and then *N*-acetylation. 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 Man_x-GlcNAc-Ins with x = 0, 1, 2, 4.

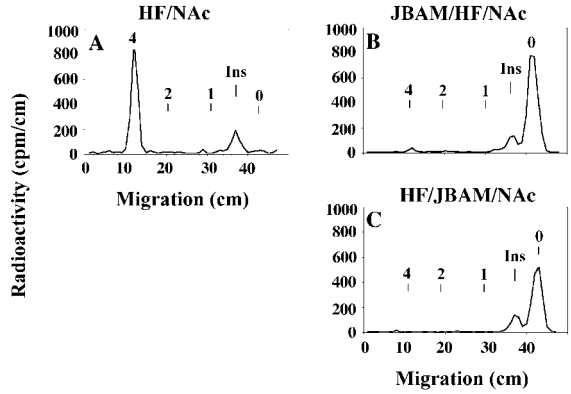

FIG. 6. **Analysis of the head group of lipid 031a using HF and JBAM.** FBY1102 cells were labeled with [^{3}H]Ins at 30 °C, lipid 031a was purified by two rounds of preparative TLC, and head groups were prepared. Head groups were subjected to the following treatments. *A*, HF followed by *N*-acetylation; *B*, JBAM followed by HF and then *N*-acetylation; *C*, HF followed by desalting, JBAM, and then *N*-acetylation. The thus generated fragments were separated as in Fig. 5.

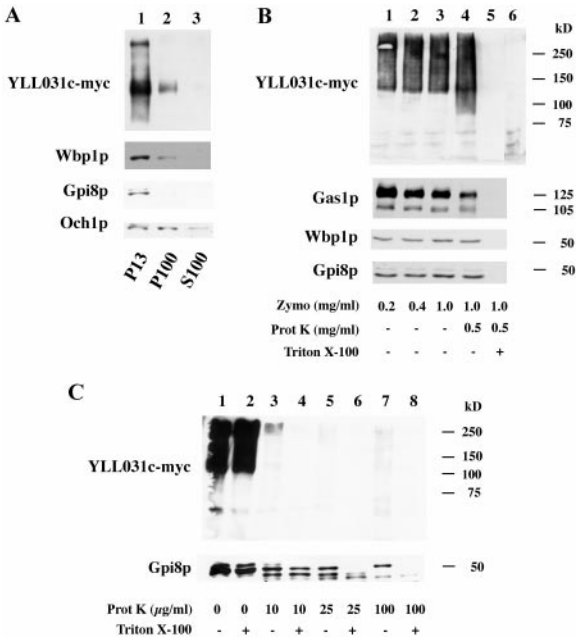

FIG. 7. **Subcellular localization of YLL031c.** *A*, FBY1107 cell lysates were subjected to differential centrifugations at 13,000 and 100,000 × *g*. These centrifugations generated pellet P13, which contains ER, plasma, and vacuolar membranes, and pellet P100, which contains Golgi membranes. The 100,000 × *g* supernatant (*S100*) was precipitated with trichloroacetic acid. *B*, FBY1107 cells were converted to spheroplasts using various concentrations of zymolyase-20T and then digested with proteinase K at the indicated concentrations. *Lane 6* shows the reactivity of the anti-Myc antibody and secondary antibody with an extract from FBY413 WT cells not harboring any Myc-tagged proteins. This control extract was prepared as described in the legend to Fig. 3. *C*, FBY1107 microsomes were sedimented at 13,000 × *g* for 15 min, and the membrane pellet was thoroughly resuspended and digested with the indicated amounts of proteinase K at 0 °C on ice for 20 min in the presence or absence of 0.5% Triton X-100. Samples were processed for SDS-polyacrylamide gel electrophoresis and Western blotting.

structure proposed above. A second, less prominent Man_2-GlcNAc-Ins peak in Fig. 5*C* could not be reduced by increasing the concentration of JBAM. This suggests the presence of a lipid having the structure Manα1–2Manα1–2[NH_2-$(CH_2)_2$-PO_4→]Manα1–6Manα1–4GlcNα1–6[acyl→]Ins-PO_4-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 Man_4-GlcN-Ins core structure (Fig. 6*A*) and that all of its four mannoses can be removed by JBAM not only after but also before HF dephosphorylation (Fig. 6, *C* and *B*). These data strongly suggest that lipid 031a has the structure Manα1–2Manα1–2Manα1–6Manα1–4GlcNα1–6[acyl→]inositol-PO_4-lipid. Thus, the GPI structures accumulating upon YLL031c depletion 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 cells containing the tagged YLL031c grew at the same rate as WT cells and were completely resistant to CFW, indicating that the tagged version of the protein is functional. As shown in Fig. 7*B*, when intact spheroplasts were treated with variable amounts of zymolyase and proteinase K, YLL031c-Myc as well as ER proteins such as Wbp1p and Gpi8p remained intact, whereas Gas1p was strongly diminished, as expected for a surface protein and as described before (8). Gpi7p could not be detected on any blot in this experiment (not shown) because of its previously reported susceptibility to zymolyase (8). When spheroplasts were prepared with Quantazyme, both YLL031c and Gpi7p were preserved, but Gpi7p was readily destroyed by proteinase K, whereas YLL031c-Myc was completely resistant (not shown). Thus, unlike Gpi7p, YLL031c-Myc does not reside at the cell surface. Differential centrifugation of microsomes indicated that YLL031c-Myc cofractions with ER markers Wbp1p and Gpi8p, since it could be found mainly in P13, which is enriched in ER and plasma membrane (Fig. 7*A*). Minor amounts were present in P100, a fraction that is enriched in

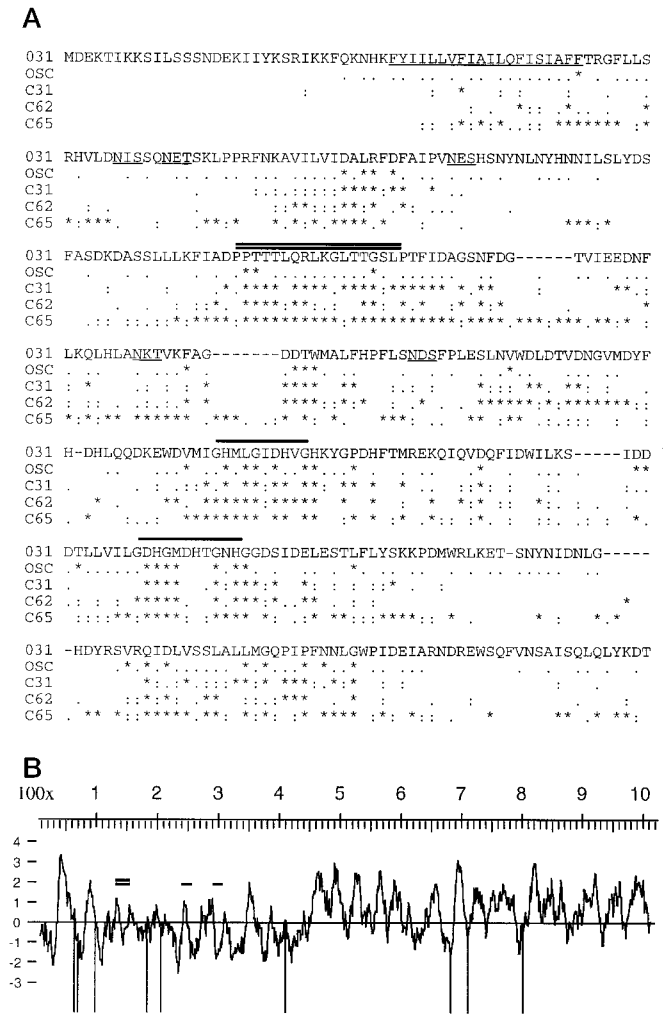

FIG. 8. **Homology of YLL031c with *GPI7* and *MCD4*.** *A*, the first 395 amino acids of YLL031c are shown. Potential *N*-glycosylation sites and the *N*-terminal hydrophobic sequence for translocation of the protein into the ER are *underlined*. Homologies with the other *S. cerevisiae* (*OSC*) genes *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*. GenBank™ accession numbers and Geninfo identifiers of sequences aligned with YLL031c were T02245 GI:7513075, AAB93646 GI:2734088, and T40030 GI:7491546; aligned with *GPI 7* were Q09782 GI:1175452 and T21487 GI:7500059, 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 YJL062w) (C62) 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 YLL031 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.

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. 7*C*). 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 hydrophobic 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 closer 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 hydrophobic 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 *GPI7* also contain the distinct, universally conserved motif PT*X*T*X*$_8$TG*X*$_2$P (*double bar above* the *sequence* in Fig. 8*A*; *stippled* in Fig. 8*B*), which is found in mammalian but also some plant and bacterial enzymes. The homology among the members of the *GPI7*/*MCD4*/YLL031c gene family encompasses 400 amino acids at the N terminus of these proteins. The family contains several motifs that are not present or are only partially conserved in phosphatases (*e.g.* H*X*LG*X*D*XX*GH and DHGM*XXX*G*X*HG (*single bar above* the *sequence* in Fig. 8*A*; *hatched* in Fig. 8*B*). These motifs fall into regions in which also

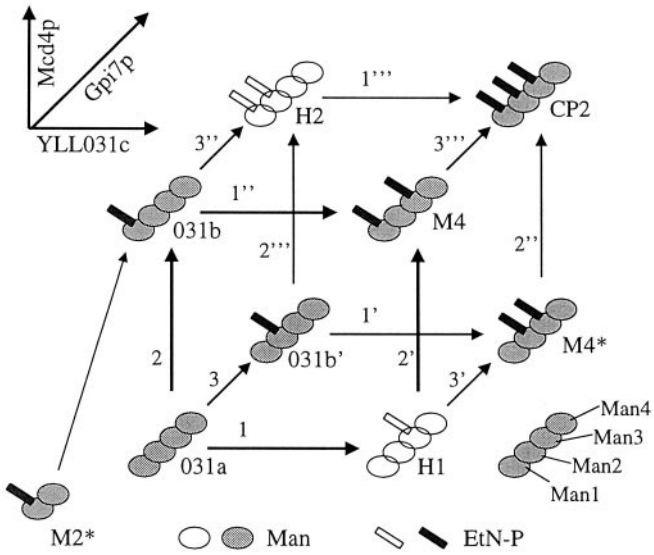

FIG. 9. **Biosynthesis of CP2: Are ethanolaminephosphates added in random order?** All theoretically possible intermediates in the biosynthesis of CP2 from 031a are placed at the corners of a cube. The six nearest pathways along the edges leading from 031a to CP2 represent all of the possible orders of the EtN-P side chain addition to the carbohydrate core. GPI structures that have actually been identified are *filled*: 031a, 031b, and 031b′ in this report; M2*, M4*, M4, and CP2 in previous publications (4, 5, 8). *H1* and *H2* indicate hypothetical structures, which have not been identified. Reactions 1, 2, and 3 are proposed to be carried out by YLL031c, Mcd4p, and Gpi7p, respectively.

the highest degree of homology is observed within each subfamily. As shown in Fig. 8*A*, the degree of consensus within each subfamily is significantly higher than between YLL031c, *GPI7*, and *MCD4* (compare *lines OSC*, *C31*, *C62*, and *C65*). Assuming that all of these genes evolved from a common ancestor, this suggests that the divergence and functional diversification of these three subfamilies occurred prior to the separation of the lineages leading to *S. cerevisiae*, *S. pombe*, *C. elegans*, and humans.

The phenotype of YLL031c-depleted cells strongly suggests that YLL031c indeed is involved in GPI anchoring. The cell wall fragility of YLL031c-depleted cells as revealed by CFW hypersensitivity is a common feature of all *gpi* mutants (33–36). Indeed, many GPI proteins of yeast are cell wall proteins or plasma membrane proteins participating in the building of the cell wall (37, 38). The appearance of abnormal GPI lipid intermediates and the specific accumulation or disappearance of mature and immature forms of GPI proteins equally point to a GPI anchoring defect.

This defect seems to be due to a defect in making GPI lipids but not in the attachment process itself. This can be concluded, since YLL031c-depleted microsomes still make some CP2 *in vitro*, but YLL031c-depleted cells do not accumulate CP2 *in vivo* as is observed in mutants that are deficient in the GPI attachment process itself (6, 7). The difficulty of YLL031c-depleted cells seems to reside in the attachment of the strategically important EtN-P to Man3, the EtN-P that will have to make the link to the protein moiety (Fig. 1). Earlier studies indicated that *GPI7* is required for the addition of an HF-sensitive substituent onto Man2, but not Man1 and Man3, and that PIG-N, the human homologue of *MCD4*, is required for the addition of EtN-P onto Man1 but not Man3. Together with the data presented here, it therefore appears reasonable to assume, as a working hypothesis, that *MCD4*, *GPI7*, and YLL031c are required for the addition of EtN-P to Man1, Man2, and Man3, respectively. Moreover, their homology with phosphodiesterases suggests that these three genes encode the corresponding EtN-P-transferases. (These transferases may be expected to bear homology with phosphodiesterases, since previous studies by the group of Anant Menon (39, 40) demonstrate that the attachment of the EtN-P to Man3 occurs by transesterification of EtN-P from phosphatidylethanolamine onto Man). A protein such as YLL031c would seem to be well suited to carry out this transfer inasmuch as its hydrophobic C-terminal domain may bind or even flip phosphatidylethanolamine, and the hydrophilic phosphodiesterase domain could operate the actual transfer reaction. Also, the subcellular localization of YLL031c in the ER is compatible with its functioning as an EtN-P-transferase, since it has been reported recently that, at least up to the stage of [NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]Ins-PO_4-lipid, GPI lipids are made in the ER (41).

The structure of the abnormal GPI lipids accumulating in various *gpi* mutant cells may not tell us in what order these transfer reactions take place in WT cells, but, if we assume that the observed GPI structures represent biosynthetic intermediates, they may tell us in what order the physiological pathway does not work. The six theoretical possibilities for the order of EtN-P additions to Man1, Man2, and Man3 starting from a Man_4-GlcN-Ins core structure are represented in Fig. 9. Pathways starting with reaction 1 (*i.e.* the addition of EtN-P by YLL031c) may not be possible, since we then would expect that *MCD4*-depleted cells or *mcd4* mutants would accumulate the theoretical structure H1 or M4*, which is not the case (Fig. 4*A*, *lane 6*) (10). Pathways using reaction 3″, leading from 031b to H2, 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″ seem unfeasible and 3 is not favored, we may predict that the physiological pathway in normal cells proceeds mainly via the only remaining way, which is 2–1″-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 is 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 Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]Ins-PO_4-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 Manα1–6[NH_2-$(CH_2)_2$-PO_4→]Manα1–4GlcNα1–6[acyl→]Ins-PO_4-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.

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Additional results

Incorporation of [^{3}H]inositol into proteins

GPI-anchored proteins are the only proteins known to be covalently linked to inositol. Thus, when GPI synthesis or attachment is impaired, moderate to severe defects in inositol incorporation into proteins are observed. We therefore tested if newly synthesized proteins in cells depleted of Gpi13p could be labeled with [^{3}H]inositol. Wild-type cells efficiently incorporated inositol into proteins (Figure 2-1, lane 4). In contrast, *gpi7Δ* cells had a partial defect in inositol incorporation (Figure 2-1, lane 1, and Benachour *et al.*, 1999), and cells depleted of Gpi13p showed a similar pattern of labeled proteins as *gpi7Δ* cells (Figure 2-1, lane 2). When Gpi13p was depleted in a *gpi7Δ* background, inositol incorporation into proteins was severely diminished (Figure 2-1, lane 3).

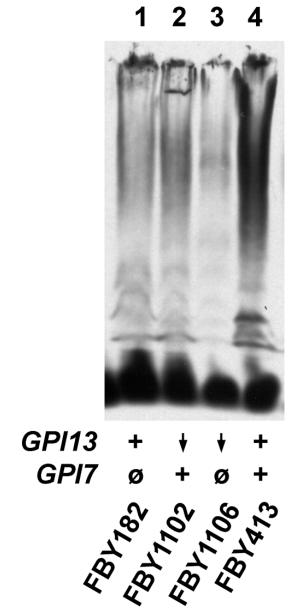

Figure 2-1. Cells depleted of Gpi13p incorporate reduced amounts of inositol into proteins.

Cells were grown overnight in SDCUA medium at 30 °C. 10 OD_{600} units of wild-type (FBY413), *Δgpi7* (FBY182), *GPI13*-depleted (FBY1102), and *Δgpi7 GPI13*-depleted (FBY1106) cells were labeled with 40 μCi *myo*-[2-[^{3}H]]inositol. Proteins were extracted and delipidated as described (Guillas *et al.*, 2000), and analyzed by SDS-PAGE/fluorography.

Myc-tagged Gpi13p is functional

As described in Flury *et al*. (2000), a 13*myc* tag was inserted in a wild-type yeast strain at the 3' end of the chromosomally encoded *GPI13* gene by homologous recombination with an insertion cassette (Longtine *et al*., 1998). A clone in which the PCR-made insertion cassette was targeted correctly, as verified by whole yeast cell PCR (Huxley *et al*., 1990), was grown on YPD plates with or without Calcofluor White (CFW). CFW is a drug that interferes with cell wall assembly and is toxic for cells having weakened cell walls, a phenomenon commonly observed in *gpi* mutants (Ram *et al*., 1994). Wild-type cells were able to grow at all dilutions irrespective of the growth medium (Figure 2-2A and B). In contrast, cells depleted of Gpi13p had a reduced growth rate on YPD plates (Figure 2-2A) and were hypersensitive to CFW (Figure 2-2B). Cells expressing the *myc*-tagged version of Gpi13p grew similar to wild-type cells on YPD medium (Figure 2-2A), and were completely resistant to CFW (Figure 2-2B), indicating that C-terminally 13*myc*-tagged Gpi13p is functional.

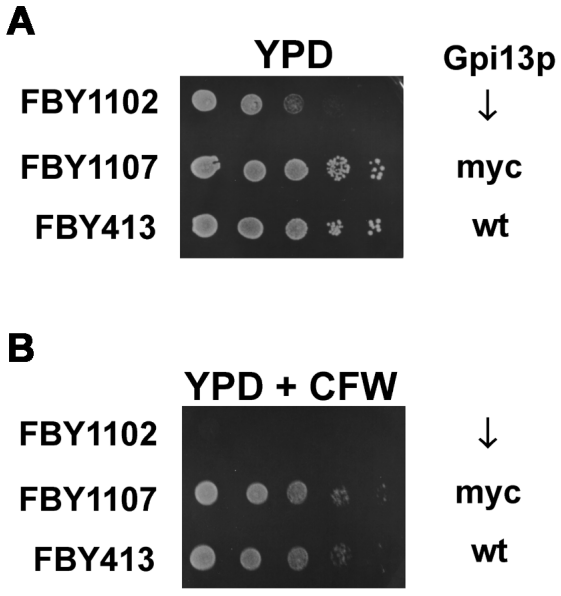

Figure 2-2. Cells expressing 13*myc*-tagged Gpi13p grow like wild-type cells.
Sequential 1:10 dilutions of cell suspensions were prepared and spotted on agar plates containing either A) YPD medium or B) YPD medium with 1.5 mg/ml CFW. Plates were photographed after 3 days at 30 °C. The amount and form of Gpi13p is indicated: ↓, depleted due to repression of *GAL1,10* promoter; *myc*, *myc*-tagged Gpi13p expressed from its promoter; wt, normal levels of Gpi13p expressed from its promoter.

Another experiment was performed in order to verify that the *myc* tag does not interfere with the function of Gpi13p. Cells expressing *myc*-tagged Gpi13p as the only source of Gpi13p were labeled with [^{3}H]Ins, lipids were extracted and analyzed by TLC. Gpi13p-depleted cells accumulated lipid 031b (Figure 2-3, lane 3), whereas cells expressing Gpi13p-*myc* had a lipid profile that was identical to wild-type (Figure 2-3, lanes 1 and 2). This result further indicated that the presence of a 13*myc* tag at the *C*-terminus of Gpi13p does not disturb the function of Gpi13p.

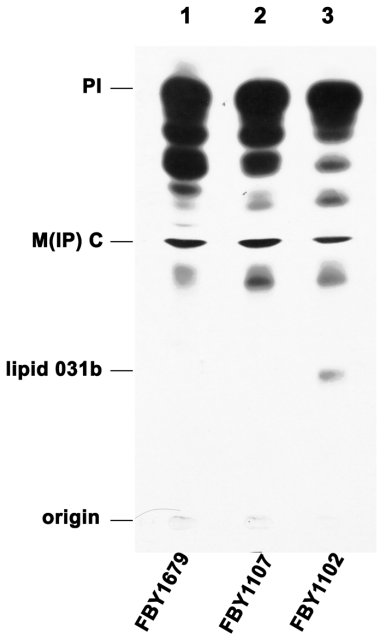

Figure 2-3. Cells expressing 13*myc*-tagged Gpi13p have the same [^{3}H]Ins-labeled lipids as wild-type cells.

Cells were grown to exponential phase in SDCUA medium and labeled with [^{3}H]Inositol (4 μCi/1-2 x 10^7 cells) at 30°C as previously described. Lipids were extracted, desalted, and analyzed by TLC using solvent 1 ($CHCl_3$/CH_3OH/H_2O 10:10:3 v/v/v) as previously described. FBY1679, wild-type; FBY1107, *GPI13-myc*; FBY1102, *pGAL1,10-GPI13*.

Membrane association of Gpi13p-*myc*

The amino acid sequence of Gpi13p predicts a protein with multiple transmembrane domains (Flury *et al.*, 2000). We therefore wanted to confirm the membrane localization of Gpi13p experimentally. Gpi13p-*myc* is associated with membranes, since it could be sedimented by ultracentrifugation of lysates at 100'000 x g for 60 min (Figure 2-4, lanes 1

and 2). Neither NaCl nor sodium carbonate at pH 11 nor urea was able to dissociate Gpi13p-*myc* from membranes, but the protein was solubilized by Triton X-100 and SDS (Figure 2-4).

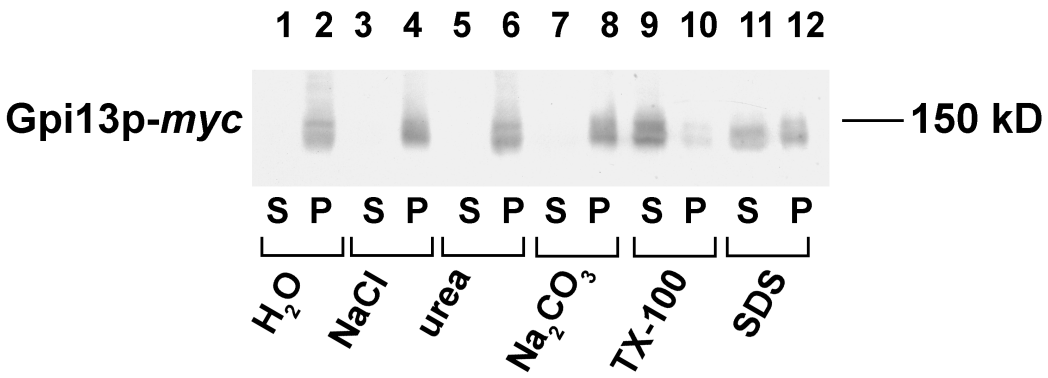

Figure 2-4. Membrane association of Gpi13p-*myc*.
Aliquots of lysate from FBY1107 cells were incubated for 40 min at 0 °C with water, 0.5 M NaCl, 0.8 M urea, 0.1 M Na_2CO_3 pH 11, 1% Triton X-100, or 1% SDS. Samples were subsequently sedimented by ultracentrifugation to get supernatant (S) and pellet (P) fractions. Samples were processed for SDS-polyacrylamide gel electrophoresis and Western Blotting with anti-*myc* antibodies. All lanes contain material derived from 6 OD_{600} of cells.

Subcellular localization of Gpi13p-*myc*

Myc-tagged Gpi13p was shown not to reside at the cell surface (Flury *et al.*, 2000). However, when looking at the immunoblots for Gpi13p-*myc* therein for the subcellular localization experiments, one can observe that material reacting with the anti-*myc* antibody is also found as a smear of high molecular mass proteins in protein samples that were denatured in "traditional" sample buffer (Fig. 7B and 7C in Flury *et al.* (2000)), whereas in samples denatured with high urea (HU) buffer (Fig. 7A therein), there is almost no such smear. We repeated the experiment shown in Figure 7B in Flury *et al.* (2000), but instead of denaturing proteins in sample buffer at 95 °C, HU buffer (8M urea, 5% SDS, 200 mM Tris-HCl pH 6.8, 20 mM EDTA, bromophenol blue, 15 mg/ml DTT) was used at 65 °C. Under these conditions, *myc*-tagged Gpi13p was found as a distinct band in protein samples denatured with HU buffer (Figure 2-5).

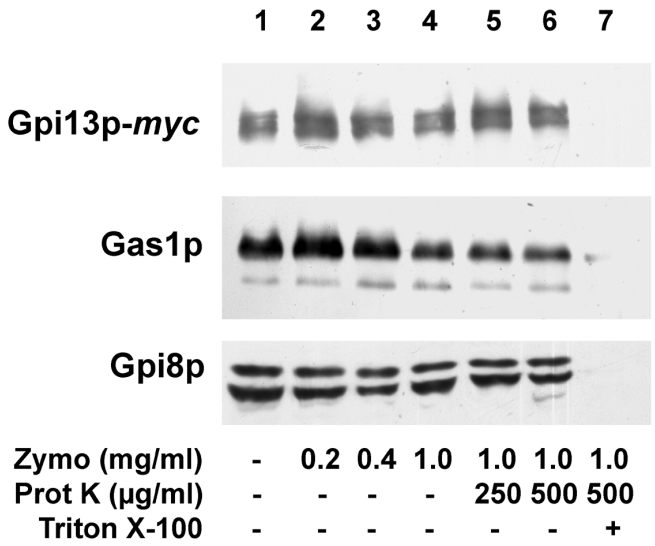

Figure 2-5. Protease treatment on intact spheroplasts.

FBY1107 cells were converted to spheroplasts using the indicated concentrations of zymolyase-20T and then digested with proteinase K or control incubated. Samples were processed for SDS-polyacrylamide gel electrophoresis and Western Blotting.

Antibodies against the hydrophilic portion of Gpi13p

In order to prepare an antibody recognizing the native form of Gpi13p, we used the same strategy as for raising of anti-Gpi7p antibodies. We chose a region comprising amino acids 122 to 320 of Gpi13p. The corresponding coding sequence was PCR-amplified from genomic DNA and cloned into the pET-15b expression vector. The His_6-tagged fusion protein was purified from bacteria and used to immunize two rabbits and to prepare an affinity column for purification of antibodies. The affinity-purified antibodies were used to detect Gpi13p in subcellular fractions obtained from wild-type cells and the results obtained were compared with data from experiments with a strain expressing 13*myc*-tagged Gpi13p. As expected, wild-type cells and cells expressing Gpi13p-*myc* showed no major difference in the localization pattern of Gpi8p and Och1p (Figure 2-6 A and B). The distribution of material reacting with anti-Gpi13p antibodies and with anti-*myc* antibodies was also very similar. However, when looking at the molecular weight markers, one can observe that the antibodies recognize bands of very different sizes. The molecular weight of the primary translation product is 136 kD for Gpi13p-*myc*, and 115 kD for untagged Gpi13p. Therefore, if our affinity-purified antibodies really recognize Gpi13p, we expected the antibodies to react with a protein migrating slightly faster than *myc*-tagged Gpi13p. As it can be seen in

Figure 2-6, this is clearly not the case. Indeed, the antibodies recognized predominantly a protein having the same localization pattern as Gpi8p and Gpi13p-*myc*, but less mobility on SDS-PAGE than Gpi13p-*myc*. This result suggests that the affinity-purified anti-Gpi13p antibodies recognize a protein unrelated to Gpi13p. The band indicated with an asterisk in Figure 2-6B is unlikely to be Gpi13p-*myc*, since a band with the same mobility was also detected by the antibody in the fraction of soluble proteins S100 (not shown).

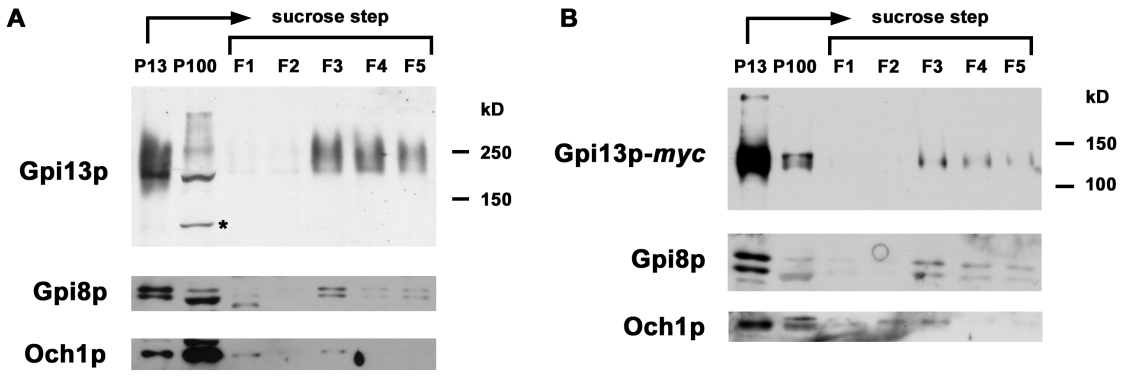

Figure 2-6. Subcellular localization of Gpi13p and Gpi13p-*myc*.
Subcellular fractionation using A) Wild-type cells, and B) cells expressing Gpi13p-*myc*. Proteins were analyzed by SDS-PAGE and Western Blotting. Equivalent amounts of proteins were loaded in all lanes. A) affinity-purified Gpi13p antibodies, and B) anti-*myc* antibodies were used to detect Gpi13p. The band indicated by an asterisk was also contained in the soluble fraction S100.

Discussion

We and Taron *et al.* (2000) have shown that *YLL031c/GPI13* encodes an essential component of the GPI biosynthetic pathway. Lipids that accumulate in cells depleted of Gpi13p have been analyzed and it was shown that these GPI intermediates lack the bridging EthN-P on the third mannose residue, suggesting that Gpi13p is the transferase that adds EthN-P to this position of the GPI core. The depletion of mature GPI-anchored proteins but not other secretory proteins in Gpi13p-depleted cells provides additional evidence that *GPI13* is involved in GPI anchor biosynthesis (Flury *et al.*, 2000). We have

shown that cells depleted of Gpi13p incorporate less inositol into proteins than wild-type cells (Figure 2-1). This defect is less severe than we would have predicted in view of the essential role of Gpi13p in GPI anchoring. Since disruption of *GPI13* is lethal, we placed the chromosomally encoded *GPI13* gene under control of the *GAL1,10* promoter and depleted the cells from Gpi13p by shifting cells from galactose- to glucose-containing medium. With this method, we could only partially deplete the cells from Gpi13p, since these cells were able to grow on glucose-containing medium, albeit at a reduced rate (Flury *et al.*, 2000). Therefore, the observed inositol incorporation into proteins in cells depleted of Gpi13p is likely due to residual expression of Gpi13p on glucose-containing medium.

Then, we demonstrated in two different experiments that insertion of a 13*myc* epitope at the *C*-terminus of Gpi13p does not interfere with its function, since cells expressing only the tagged form of Gpi13p grew like wild-type cells and did not accumulate GPI intermediates (Figures 2-2 and 2-3). The localization experiments of Gpi13p using commercially available anti-*myc* antibodies were based on the finding that *myc*-tagged Gpi13p is functional. Therefore, the investigations on the subcellular localization performed in Flury *et al.* (2000) as well on the membrane association (Figure 2-4) of Gpi13p were performed in a strain expressing Gpi13p-*myc*.

We also provided experimental evidence that Gpi13p is a membrane-anchored protein, since it could only be solubilized by treating cell lysates with detergents such as SDS and Triton X-100. As shown in Flury *et al.* (2000) and in Figure 2-5, treatment of intact spheroplasts with protease did not diminish ER-resident proteins, whereas proteins located on the cell surface were digested. Gpi13p-*myc* as well as the ER protein Gpi8p and the 105 kD ER-form of Gas1p remained intact when spheroplasts were treated with protease, whereas the 125 kD mature form of Gas1p was strongly diminished, as expected for a protein residing at the surface. This result clearly shows that Gpi13p-*myc* is not localized at the plasma membrane. Consistent with this result, the mammalian homologue of Gpi13p, PIG-O, was localized in the ER (Hong *et al.*, 2000).

The production of antibodies against a portion of Gpi13p did not give satisfying results, since affinity-purified antibodies reacted with material that was unlikely to be Gpi13p. Indeed, the antibody recognized a smear that had significantly less mobility on SDS-PAGE than we expected from the amino acid sequence of Gpi13p, and also less mobility than the material recognized by the anti-*myc* antibody (compare Figure 2-6 A and B). We conclude

that our affinity-purified anti-Gpi13p antibody recognized a protein other than Gpi13p. When one compares the sequence of the recombinant proteins used to raise anti-Gpi7p antibodies (Benachour *et al.*, 1999) and these anti-Gpi13p antibodies, one realizes that in both cases a large fragment in the hydrophilic region of the protein was chosen. And as shown in a sequence alignment of Gpi13p and Gpi7p (Flury *et al.*, 2000), the homologous stretches of Gpi13p and Gpi7p are located in this hydrophilic region. Therefore it might be conceivable that our anti-Gpi13p antibodies crossreact with Gpi7p or with Mcd4p. The fragment of Gpi13p used to raise these antibodies contains several regions of up to 6 amino acids that are identical with stretches from Gpi7p, and regions with up to 4 amino acids identical with Mcd4p. However, a control experiment has shown that protein extracts of a *Δgpi7* strain also contained material reacting as a smear with affinity-purified anti-Gpi13p antibodies, suggesting that the smear is not Gpi7p, but rather another protein. The results presented here showing that a strain expressing *13myc*-tagged Gpi13p is functional suggest that working with this strain and anti-*myc* antibodies are a good alternative to the use of anti-Gpi13p antibodies described here which might also recognize the Gpi13p-homologues. Nevertheless, we are currently raising antibodies against a 16 amino acid peptide (SKKPDMWRLKETSNYN) in the hydrophilic region of Gpi13p having no homology to Gpi7p or Mcd4p. Once we have obtained these new antibodies, we will reinvestigate the localization of Gpi13p in order to rule out the possibility that insertion of the *myc* epitope tag at the C-terminus of Gpi13p causes mislocalization. It has been reported that epitope tagging of the ER-localized protein Ste14p at either end caused mislocalization to the Golgi apparatus, even though the N-terminally HA-tagged Ste14p was functional (Romano *et al.*, 1998).

3. Characterization of Gpi7p

Summary

GPI anchoring is a post-translational modification reaction whereby preassembled GPI anchors are transferred to proteins bearing a *C*-terminal GPI signal sequence in the ER. The GPI anchor precursors are synthesized by stepwise addition of sugars and ethanolaminephosphate to phosphatidylinositol. *Saccharomyces cerevisiae* mutants deficient in the GPI biosynthetic pathway are a convenient tool to identify genes involved in GPI anchoring. The *gpi7* mutant was identified in a screen for *Saccharomyces cerevisiae* strains deficient in the surface expression of the GPI-anchored proteinα-agglutinin (Benghezal *et al.*, 1995). The corresponding GPI7 gene was cloned by complementation. It is a non-essential gene encoding a protein with multiple predicted transmembrane domains. *Δgpi7* cells accumulate a GPI precursor lacking a previously unrecognized HF-sensitive substituent on the second mannose residue of the GPI core, suggesting that Gpi7p may be involved in addition of this side chain. The objective of this thesis work in the context of *GPI7* was the characterization of Gpi7p. Using polyclonal antibodies raised against its hydrophilic portion, we have shown that Gpi7p is localized at the cell surface.

Publication: 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

I have carried out the experiments showing that Gpi7p is localized at the cell surface, and I have prepared [^{3}H]mannose-labeled CP2 and M4 glycolipids.

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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 Δ*gpi7*. 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 phosphodiesterases suggesting that Gpi7p itself is the transferase adding a side chain to the α1,6-linked mannose of the GPI core structure.**

Glycosylphosphatidylinositol (GPI)[1]-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 proteins (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 NH_3 followed by HF treatment, used to hydrolyze selectively the phosphodiester bonds (Fig. 1), yielded the same Man_4-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 FBY11 (*MAT***a** *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1*), FBY15 (*MATα ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi7-1*), W303-1B (*MATα ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11,15*),

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; ASAM, *A. satoi* α-mannosidase; CP, complete precursor; DAG, diacylglycerol; DHS, dihydrosphingosine; EtN-P, ethanolamine phosphate; GPI-PLD, GPI-specific phospholipase D; Ins, *myo*-inositol; JBAM, jack bean α-mannosidase; Man, mannose; ORF, open reading frame; pC1 and pC2, protein-derived Ceramides 1 and 2; pG1 protein-derived Glycerophospholipid 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.

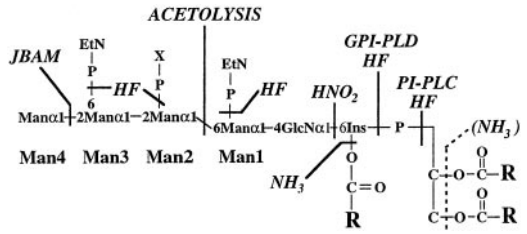

FIG. 1. **Presumed structure of the complete yeast precursor glycolipid CP2.** Relevant cleavage procedures are indicated. Man1, Man2, Man3, and Man4 designate the α1,4-linked, α1,6-linked, and α1,2-linked mannoses (Man). *X* indicates an HF-sensitive group that is not yet defined chemically. *R,* alkyl; *P,* phosphate.

X2180-1A (*MAT**a** lys*$^-$), FBY122 (MAT***a*** *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1 gpi7-1*), FBY182 (*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::KanMX4*), HMSF176 (*MAT**a** sec18-1*), FBY49 (*MAT**a** sec18-1 gpi7::KanMX4*), C4 (*MAT**a** ura3-52 leu2-3,112 pmi40*), HMSF331 (*MAT**a** sec53-6*), LB2134-3B (*MAT**a** mnn9*), and YNS3-7A (*MAT**a** ura3 his*$^-$ *mnn1 och1::LEU2*). Diploid strains were FBY118 (*MAT**a**/α ade2-1/ade2-1 ura3-1/ura3-1 leu2-3, 112/leu2-3,112 TRP1/trp1-1 his3-11,15/his3-11,15 LYS/lys*$^-$), FBY40 (MAT***a***/*α ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 TRP1/trp1-1 his3-11,15/his3-11,15 LYS/lys*$^-$ *GPI7/gpi7-1*), and FBY43 (*MAT**a**/α ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 TRP1/trp1-1 his3-11,15/his3-11,15 LYS/lys*$^-$ *gpi7::KanMX4/gpi7::KanMX4*). Maintenance and growth conditions have been described (19). The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm, and one A_{600} unit of cells corresponds to 1–2 $\times$ 10^7 cells depending on the strain. *Escherichia coli* strains were HB-101, XL1 blue, and M15 [pREP4] (Qiagen).

Materials were obtained from the sources described recently (22). Cysteamine was from Sigma; [^{3}H]dihydrosphingosine was synthesized as described (24); myriocin was a kind gift of Dr. N. Rao Movva (Novartis, Basel, Switzerland); antibodies to Och1p, alkaline phosphatase, Kex2p, and Wbp1p were kindly donated by Dr. Y. Jigami, National Institute of Bioscience and Human Technology, Ibaraki 305, Japan; Dr. S. Emr, Howard Hughes Medical Institute, University of California, San Diego; Dr. R. Fuller, University of Michigan Medical Center, Ann Arbor, MI; and Dr. M. Aebi, Mikrobiologisches Institut, ETH Zürich, Switzerland, respectively.

Cloning, Partial Sequencing, and Disruption of GPI7—The *GPI7* gene was cloned by complementation of the ts growth phenotype of the *gpi7-1/gpi8-1* double mutant as described (19). The three plasmids complementing *gpi7-1* contained a 4.3-kb common DNA restriction fragment that was partially sequenced by the dideoxy sequencing method (25). The complementing insert *Sph*I/*Ssp*I of 3.6 kb was cloned into the *Sph*I/*Sma*I-digested YEp352 multicopy vector (26) or YCplac33 single copy vector (27) to generate pBF41 (Fig. 3*C*) and pBF43, respectively.

One step disruption of *GPI7* was done as described (28). Briefly, the 1.5-kb long KanMX4 module was PCR-amplified by using pFA6a-KanMX4 as template and the following two adapter primers: GPI7-forwards (5′-CTTCACCAAGTTAGCAAG**ATG**AACTTGAAGCAGTTC-ACGTGCCTcgatgaattcgagctc-3′) with 17 nucleotides (nt) of homology to the pFA6a-KanMX4 multiple cloning site (in *lowercase*) and 43 nt of homology to *GPI7* (in uppercase) starting 18 nt upstream of the start codon (*bold*); GPI7-backwards (5′-ATCAAGAGCGCAAAGGAGGGCC-AATTCAGGTAACCAGCCATTCAcgtacgctgcaggtcgac-3′) with 18 nt of homology to the pFA6a-KanMX4 multiple cloning site (*lowercase*) and 44 nt of homology to the ORF of *GPI7* in the region immediately upstream of the stop codon. This PCR DNA fragment was used to transform the diploid strain FBY118, homozygous for *GPI7*, and FBY40, a heterozygous *gpi7-1/GPI7* strain. The correct targeting of the PCR-made KanMX4 module into the *GPI7* locus in geneticin-resistant clones was verified by PCR with whole yeast cells using primers GPI7-plus (5′-GTTCATCTACCACGCAC-3′) starting 36 nt upstream of the ATG, GPI7-minus (5′-GACCCAAGTAATGCAGG-3′) starting 631 base pairs downstream of the ATG and the K2 primer of the KanMX4 module (5′-GTATTGATGTTGGACG-3′).

Purification of Recombinant His-tagged Gpi7p and Antibody Production—Plasmid pBF41 (Fig. 3*C*) was digested with *Bst*YI and *Eco*RV to generate a 633-base pair fragment of *GPI7*. This fragment was inserted into the multiple cloning site of the bacterial expression vector pQE-30 (Qiagen) digested with *Bam*HI/*Sma*I thus generating the plasmid pBF402. This plasmid was used to transform the *E. coli* strain M15[pREP4]. Expression of the recombinant protein was induced with isopropyl-1-thio-β-D-galactopyranoside and purified on a nickel-nitrilotriacetic acid-agarose column (Qiagen) under denaturing conditions according to the manufacturer's instructions. A polyclonal antiserum was raised against this Gpi7p fragment by repeated intramuscular injections of 100 μg of recombinant protein into a rabbit. Ten mg of the recombinant protein were coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions, and antiserum was affinity purified as described (29).

Membrane Association, Protease Sensitivity, and Cellular Localization of Gpi7p—The nature of the association of Gpi7p with the membrane was determined following a previously described protocol (30) except that the EDTA concentration in buffer G was increased from 2 to 20 mM. Gpi7p protease sensitivity was examined by proteinase K digestion of microsomes essentially as described (31). Briefly, 100 A_{600} of washed W303 cells were resuspended in 1 ml of lysis buffer (20 mM HEPES, pH 7.5, 500 mM sucrose, 3 mM magnesium acetate, 20 mM EDTA, 1 mM dithiothreitol) and were lysed by agitation with glass beads at 4 °C. The homogenate was centrifuged for 5 min at 600 $\times$ *g* to remove unbroken cells, and the supernatant was centrifuged for 15 min at 13,000 $\times$ *g*. The membrane pellet was resuspended in 240 μl of the same lysis buffer and split into 6 aliquots of equal size. Aliquots of microsomes were incubated for 20 min on ice with or without 0.5% Triton X-100 and proteinase K. Digestions were stopped by addition of phenylmethylsulfonyl fluoride (final concentration 4 mM, added from a 200 mM stock in ethanol) and kept on ice for an additional 10 min before being boiled in sample buffer (32). The subcellular localization of Gpi7p was determined essentially as described (33). Briefly, 500 A_{600} units of mid-log phase W303-1B cells were broken by agitation with glass beads in 200 mM sorbitol, 25 mM PIPES, pH 6.8, 50 mM KCl, 5 mM NaCl, 10 mM EDTA, 10 mM NaN_3/NaF, 1 mM phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and antipain, each at 30 μg/ml). After removal of the unbroken cells the homogenate was centrifuged for 10 min at 8,000 $\times$ *g* at 4 °C to generate pellet P8 and supernatant S8. S8 was divided in two and either precipitated by the addition of trichloroacetic acid to 10% or centrifuged at 100,000 $\times$ *g* for 1 h to generate pellet P100. For zymolyase treatment the cells were washed and resuspended at 50 A_{600} units/ml in zymolyase buffer (1.2 M sorbitol, 50 mM K_2HPO_4 pH 7.5, 40 mM 2-mercaptoethanol, 20 mM EDTA, 10 mM NaN_3, 10 mM NaF) containing zymolyase 20T. After a 40-min incubation at 30 °C, cells were placed on a cushion of 1.5 M sorbitol, 50 mM K_2HPO_4, pH 7.5, 20 mM EDTA, 10 mM NaN_3, 10 mM NaF and were centrifuged. For Western blotting, all the samples were denatured during 5 min at 95 °C in reducing sample buffer and run on a 6, 7.5, or 10% SDS-PAGE for detection of antigens, respectively (32). Western blotting was carried out with anti-Wbp1p, anti-alkaline phosphatase, anti-Gas1p, or anti-Kex2p antisera or with affinity purified anti-Gpi7p or anti-Och1p antibodies, always using the chemiluminescence ECL kit from Amersham Pharmacia Biotech, Buckinghamshire, UK.

Labeling of Cells—Previously described procedures were used to label cells with [2-^{3}H]Man (23), [4,5-^{3}H]DHS, or [2-^{3}H]Ins (24) and to label microsomes with UDP-[^{3}H]GlcNAc (22). Delipidated protein extracts for SDS-PAGE and lipid extracts were made as described (24). Lipid extracts were analyzed by ascending TLC using 0.2-mm thick silica gel 60 plates with the solvent 1 (chloroform/methanol, 0.25% KCl in water, 55:45:10, v/v) or solvent 2 (chloroform/methanol/water, 10:10:3, v/v). Radioactivity was detected and quantitated by one- and two-dimensional radioscanning (LB 2842; Berthold AG, Regensdorf, Switzerland). TLC plates were sprayed with EN^{3}HANCE and exposed to film (X-Omat; Eastman Kodak Co.) at −80 °C.

Analytical Methods—Lipid extracts were deacylated with NaOH (34) and treated with JBAM (35) as described. For GPI-PLD treatment lipid extracts were dissolved in 20 mM Tris-HCl, pH 7.4, 0.1 mM $CaCl_2$, 20% 1-propanol. Incubations were for 12 h at 37 °C. All treated lipid extracts were desalted by partitioning between *n*-butyl alcohol and an aqueous solution of 0.1 mM EDTA, 5 mM Tris-HCl, pH 7.5, and back extraction of the butanol phase with water before TLC (23). Lipids were treated with methanolic NH_3 to remove the acyl group on Ins (36) and cleaved using nitrous acid (37) as described. Lipids were purified by preparative TLC on 0.2-mm thick Silica Gel 60 plates (Merck, Germany) in solvent 2. Radioactive spots were localized by radioscanning, scraped, and eluted with solvent 2. A second run on TLC was done to obtain radiochemically pure M0, M4, and CP2.

Soluble head groups were obtained from lipids through GPI-PLD treatment done as above, followed by limiting methanolic NH_3 deacylation (36). Non-hydrolyzed GPIs were removed by butanol extraction (23). The water-soluble head groups were treated with JBAM (0.5 units) or ASAM (5 microunits) as described (38). HF dephosphorylation was

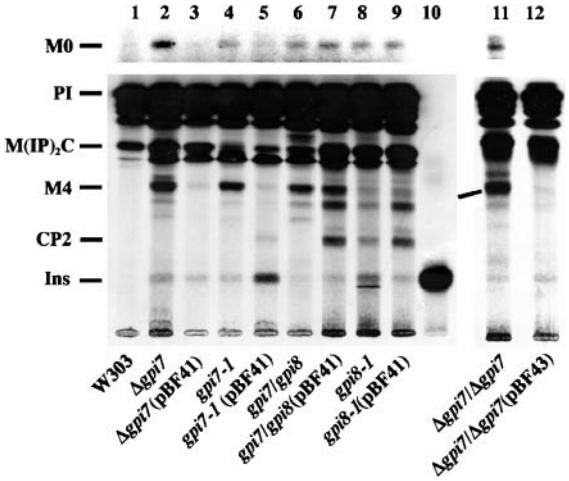

FIG. 2. **Complementation of *gpi7* mutants by YJL062w.** Exponentially growing cells were radiolabeled at 37 °C with [^{3}H]Ins (2 μCi/A_{600}), and desalted lipid extracts were analyzed by TLC (solvent 2) and fluorography. The same amount of radioactivity was spotted in each lane. *M(IP)$_2$C,* inositol phosphomannosylinositol phosphoceramide. The upper part of the fluorogram was scanned at higher sensitivity to bring into view the faint bands of *M0*. *Lane 10* contains [^{3}H]Ins. Samples still contain residual amounts of free [^{3}H]Ins after extraction into butanol. The band migrating between CP2 and M4 in *gpi8-1* is a GPI-PLD-, mild base-, and JBAM-sensitive GPI intermediate containing the Man$_4$-GlcN-Ins core,[2] but the structural differences between this species and CP2 or M4 have not been identified.

done as described (39). The generated fragments were analyzed by paper chromatography in methylethyl ketone/pyridine/H_2O (20:12:11) as described (39). Before paper chromatography the products were *N*-acetylated and desalted over mixed-bed ion exchange resin AG-501-X8 (Bio-Rad) unless indicated otherwise (34). Acetolysis was done as described (40). Radiolabeled Man$_x$-GlcNAc-[^{3}H]Ins (x = 1, 2, 3, 4) chromatography standards (Figs. 5 and 6, standards *1–4*) were generated through fragmentation of [^{3}H]Man-labeled head groups of CP2, isolated from *pmi40* by acetolysis then HF, HF then ASAM, JBAM then HF, and HF treatments, respectively. The GlcNAc-[^{3}H]Ins standard (Figs. 5 and 6, standard *0*) was generated by HF treatment of the [^{3}H]Ins-labeled head group of M0, obtained from *sec53* cells. All standards were *N*-acetylated. Dionex HPLC analysis of non-dephosphorylated head groups was done exactly as described (23). Anchor peptides were prepared from labeled proteins as described (23).

Limiting HF Treatment of Head Groups—For limiting HF treatment, aliquots of radiolabeled head groups derived from CP2 and M4 and prepared as above were dephosphorylated with 50 μl of 48% aqueous HF at 0 °C as described (38) for 0–28 h. After neutralization with saturated LiOH, samples were desalted by gel filtration through an 8-ml Sephadex G-10 (Amersham Pharmacia Biotech) column. Samples were then dried in the Speed-Vac and treated with JBAM prior to complete HF dephosphorylation (60 h, 0 °C). Samples were neutralized again with LiOH and *N*-acetylated. Aliquots were dried and then directly applied to Whatman paper No. 1M and analyzed by descending chromatography as described above.

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, Gpi7p 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).

[2] I. Flury, unpublished observations.

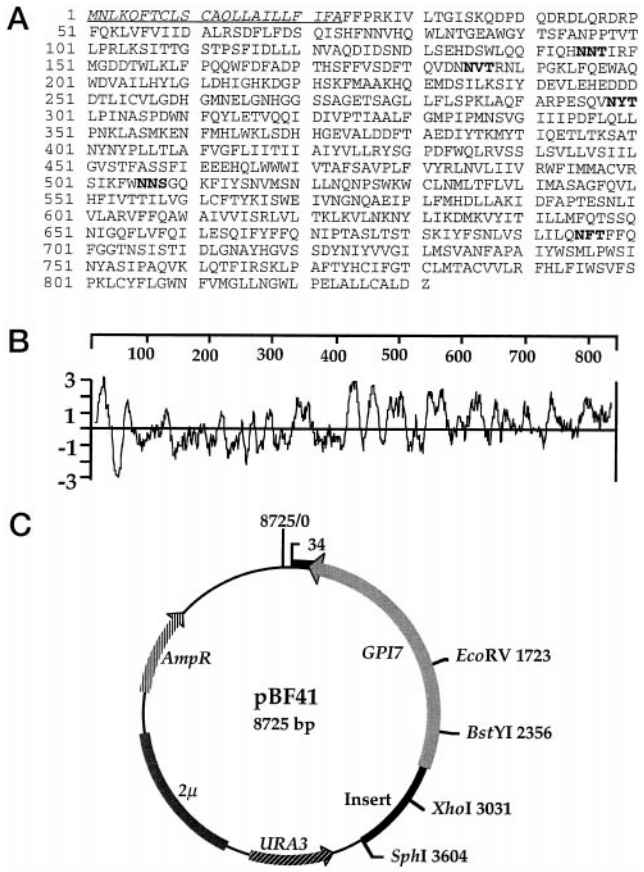

FIG. 3. **Sequence of *GPI7*.** *A,* potential *N*-glycosylation sites are shown in *boldface*. The predicted signal peptide is *underlined*. *B* shows a hydrophobicity plot according to Kyte and Doolittle, in which the hydrophobic sequences get a positive score. *C* shows the restriction map of pBF41. The 3.6-kb *insert* (34–3604) contains *GPI7* (= YJL062w, *broad arrow*). The *Eco*RV/*Bst*YI fragment was expressed in bacteria to get antigen for raising antibodies.

These clones were labeled with *myo*-[^{3}H]inositol ([^{3}H]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. 3*C*) 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, 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/YJL062 heterozygotes sporulated readily and ΔYJL062 spores germinated normally. In accordance with previous results on *gpi7* mutants (21), growth of ΔYJL062 (= Δ*gpi7*, 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

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 GlcNα1,6(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 geneticin-resistant transformants. The verified deletants were sporulated, and a total of 26 complete tetrads was labeled with [^{3}H]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. 4*A, 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 NH_3, and then subjected to several treatments as indicated at the *top* of Fig. 5, *A–D*. The *N*-acetylated fragment comigrated with the Man_4-GlcNAc-Ins standard (Fig. 5*A*). When treated with JBAM before HF, the resulting *N*-acetylated fragment comigrated with the Man_3-GlcNAc-Ins standard, clearly indicating the presence of a blocking group on Man3 (Fig. 5*B*). The blocking group on Man3 was HF-sensitive, since JBAM done after HF produced a fragment comigrating with GlcNAc-Ins (Fig. 5*C*). *Aspergillus satoi* α-mannosidase (ASAM), a linkage-specific α1,2-exomannosidase, when used after HF treatment, produced Man_2-GlcNAc-Ins (Fig. 5*D*). 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-GlcNα1,6Ins 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

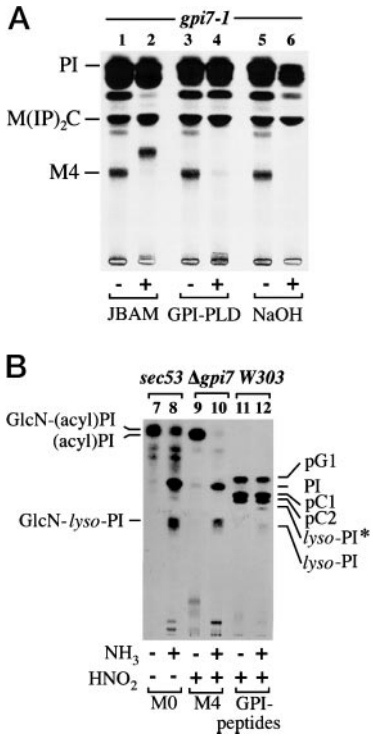

FIG. 4. **Characterization of M4.** *A, gpi7-1* cells were preincubated at 37 °C for 20 min and were labeled with [^{3}H]Ins (2 $\mu Ci/A_{600}$). Lipid extracts were treated (+) or control incubated (−) with either JBAM or GPI-PLD or were deacylated by mild base treatment (NaOH). Desalted products were analyzed by TLC (solvent 2) and fluorography. *B, sec53* and Δ*gpi7* were preincubated and labeled with [^{3}H]Ins at 37 °C (5 $\mu Ci/A_{600}$), and lipid extracts were treated with PI-specific phospholipase C to get rid of labeled PI, and the GPI intermediates M0 and M4 were purified from *sec53* and Δ*gpi7,* respectively, by two rounds of preparative TLC. Anchor peptides were prepared from [^{3}H]Ins-labeled W303 in the experiment described in Table I. M4 and anchor peptides were treated with HNO_2 to liberate the *acyl*-[^{3}H]Ins-P-lipid and [^{3}H]Ins-P-lipid moieties, respectively (Fig. 1). Samples were incubated with (+) or without (−) methanolic NH_3 to remove the acyl from the Ins, desalted and separated by TLC (solvent 1), and processed for fluorography. pG1, protein-derived glycerophospholipid 1; pC1 and pC2, protein-derived ceramides 1 and 2, see Sipos *et al.* (41). Other results of this same experiment were described before (Ref. 41, therein Fig. 2).

phosphodiesters 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 [^{3}H]Ins-labeled M4 was first cleaved by acetolysis, 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-[^{3}H]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 acetolysis, 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 Man_4-GlcN-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

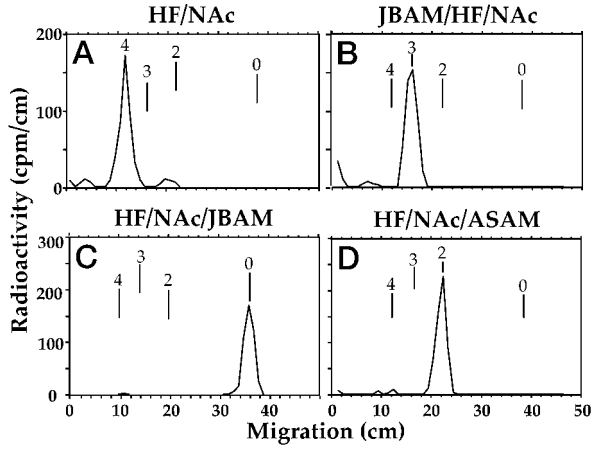

FIG. 5. **Analysis of the head group of M4 using HF, JBAM, and ASAM.** Δ*gpi7* was labeled with [^{3}H]Ins at 37 °C; M4 was purified and used to prepare head groups. Head groups were subjected to HF and *N*-acetylation (*A*); JBAM, then HF, then *N*-acetylation (*B*); HF, then *N*-acetylation, then desalting, then JBAM (*C*); HF, then *N*-acetylation, then desalting, then ASAM (*D*). The thus generated fragments were separated by paper chromatography, and radioactivity contained in 1-cm wide strips was determined through scintillation counting. The position of standards run in parallel on the same paper are indicated: *2–4,* Man_x-GlcNAc-Ins with $x = 2$, 3, or 4. *0,* GlcNAc-Ins.

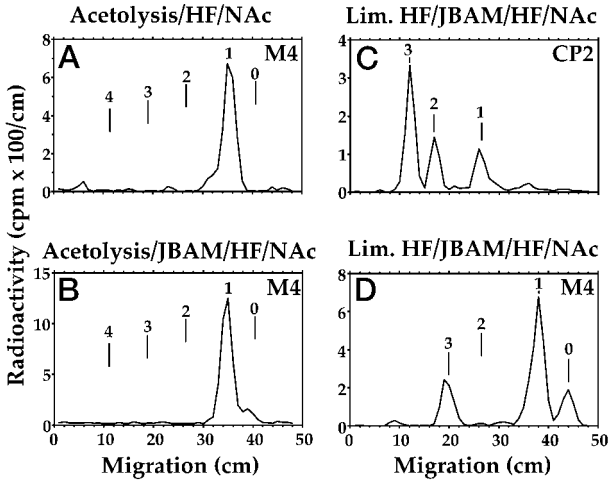

FIG. 6. **M4 of Δ*gpi7* contains an HF-sensitive substituent on Man1 but lacks an HF-sensitive substituent on Man2.** CP2 and M4 head groups were obtained from [^{3}H]Man labeled *pmi40* and [^{3}H]Ins labeled Δ*gpi7,* respectively. *A* and *B*, head groups of M4 were subjected to acetolysis and then either treated with JBAM (*B*) or left untreated (*A*). Finally all products were dephosphorylated with HF, *N*-acetylated, and analyzed by paper chromatography. *C* and *D*, head groups were treated for 12 h with HF, desalted, treated with JBAM, treated with HF for 60 h, *N*-acetylated, and finally analyzed by paper chromatography. Standards *0–4* are Man_x-GlcNAc-Ins ($x = 0, 1, 2, 3, 4$). Free [^{3}H]Man ran out of the paper shown in *C*.

HPLC (data presented above and in Refs. 22 and 23). We thus hypothesized that CP2 may contain either additional HF-sensitive groups on GlcN, Man1, Man2, or Man3 or may contain additional groups linked through the amino group of the EtN-P on M1. Of these several theoretical possibilities, the only ones that have been documented in other organisms are the Man1-P group on the GlcN in *Paramecium aurelia* (43) and the EtN-P group on Man2 in several mammalian GPI proteins, *e.g.* human erythrocyte acetylcholinesterase (44), CD52-II (45), and bovine liver 5′-nucleotidase (46). We used limiting HF treatment to test specifically if CP2 contains an HF-sensitive group on Man2. If we assume that during HF treatment the EtN-Ps are hydrolyzed in a random order, we may expect to find some reaction intermediates lacking the HF-sensitive group on 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 Man_2-GlcN-Ins and Man_1-GlcN-Ins fragments, respectively. For preliminary tests, CP2 head groups were first treated with HF for 0.5, 1, 3, 9, 12, 18, or 28 h, then with JBAM, and finally with HF for 60 h. These experiments showed that both Man_2-GlcN-Ins and Man_1-GlcN-Ins fragments became visible after 1 h of limiting HF treatment, peaked at 12 h, and remained detectable at all time points up to 28 h. In quantitative terms it appeared that Man_3-GlcN-Ins $>$ Man_2-GlcN-Ins $\geq$ Man_1-GlcN-Ins at all time points. Importantly, treatment of the head group of CP2 with HF for 12 h yielded substantial amounts of Man_2-GlcN-Ins and Man_1-GlcN-Ins (Fig. 6*C*), whereas the identical treatment performed with the head group of M4 only yielded Man_1-GlcN-Ins but no Man_2-GlcN-Ins (Fig. 6*D*). This result is compatible with the idea that Δ*gpi7* 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. 4*A, 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 HNO_2 as described recently (41). As shown in Fig. 4*B*, the treatment of purified M4 by HNO_2 produced a very hydrophobic species, which migrates very closely to M0, *i.e.* the GlcN(acyl→)Ins-P-DAG accumulating in *sec53* (Fig. 4*B, 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 NH_3 produced PI and lyso-PI (Fig. 4*B, lane 10*). This PI was compared with pG1, the PI species obtained by HNO_2 treatment of protein-bound GPI anchors from the corresponding wt strain (Fig. 4*B, 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 NH_3 treatment of anchor peptides (Fig. 4*B, 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 Δ*gpi7* the recently identified GPI intermediates that are obscured in TLC by PI and inositol phosphoceramides (Ref. 41, therein Fig. 6*A*), 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 Δ*gpi7* 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-[^{3}H]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 Δ*gpi7*

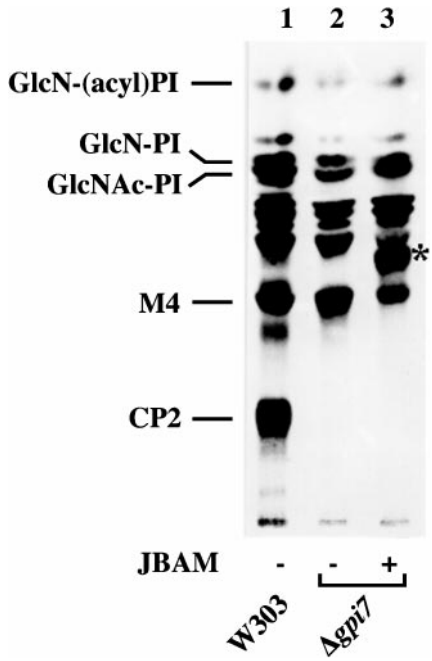

FIG. 7. **Microsomes of Δ*gpi7* synthesize M4 and not CP2.** Microsomes of W303 wild type or Δ*gpi7* were incubated with 6 μCi of UDP-[^{3}H]GlcNAc, GDP-Man, tunicamycin, and ATP for 1 h at 37 °C as described (22). The glycolipid products were extracted and then run on TLC with solvent 2. The extract 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.

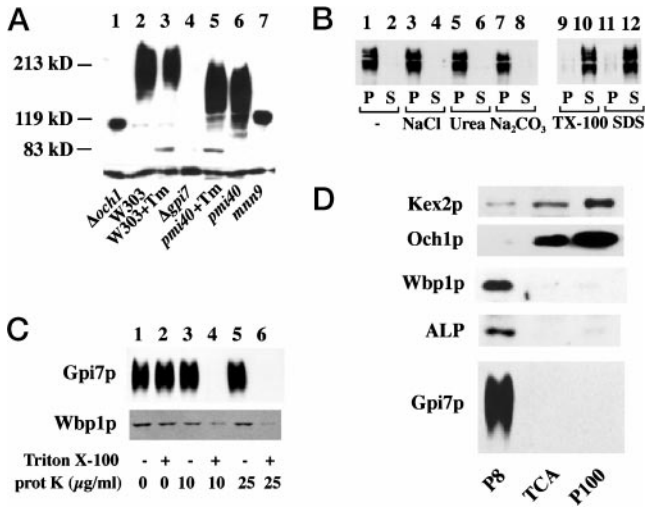

FIG. 8. **Membrane association, orientation, and localization of Gpi7p.** *A,* exponentially growing cells were broken with glass beads in TEPI buffer as described (19); lysates were centrifuged at 10,000 × *g* for 15 min at 4 °C, and microsomal pellets were processed for SDS-PAGE. Cells in *lanes 3* and *5* had been grown in 20 μg/ml tunicamycin (*Tm*) 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 M NaCl, 0.8 M urea, 1% Triton X-100 (*TX-100*), 0.1 M Na_2CO_3, 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 A_{600} of cells except for *lanes 1* and *7* of *A* which contain 0.3 A_{600}.

microsomes, they reproducibly made all the normal intermediates down to a band comigrating with M4 of [^{3}H]Ins-labeled Δ*gpi7* 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 Δ*gpi7* 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 part of *GPI7* (Fig. 3*B*). As shown in Fig. 8*A*, 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. 8*A, 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 Δ*och1/mnn1* or *mnn9* which are totally or partially deficient in the elongation of *N*-glycans in the Golgi, Gpi7p has an estimated mass of 108 and 115 kDa, respectively (Fig. 8*A, 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. 8*A, 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. 8*A, 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 60 min (Fig. 8*B, lanes 1* and *2*). Gpi7p was neither dissociated from membranes by NaCl nor sodium carbonate at pH 11, nor urea, but it was efficiently solubilized by Triton X-100 or SDS (Fig. 8*B*). The presence of an N-terminal signal sequence and the large amount of *N*-glycans suggested that the hydrophilic N-terminal part of the protein, which contains 3 of the 5 potential *N*-glycosylation sites, would reside on the lumenal or ectocytoplasmic side of the membrane. We tried to confirm this orientation by protease protection assays on microsomes. As shown in Fig. 8*C*, neither Gpi7p nor Wbp1p, which was used as a control, were degraded by proteinase K unless microsomes were permeabilized with Triton X-100. Wbp1p has been demonstrated to be a lumenal ER protein (49), and our result thus shows that the N-terminal hydrophilic part of Gpi7p is not accessible to protease in these microsomes. It is noteworthy that proteinase K did not reduce the molecular mass of Gpi7p, thus indicating that the cytosolic loops between the predicted transmembrane domains of the C-terminal part of Gpi7p are not accessible to proteinase K in native microsomes.

The cellular localization of Gpi7p was investigated by subcellular fractionation as shown in Fig. 8*D*. Differential centrifugation at 8,000 and 100,000 × *g* for 10 and 60 min, respectively, achieved satisfactory separation of the Golgi markers Kex2p and Och1p from the ER marker Wbp1p and from the vacuolar alkaline phosphatase. We were concerned that the relative amounts of these proteins in the 100,000 × *g* pellet

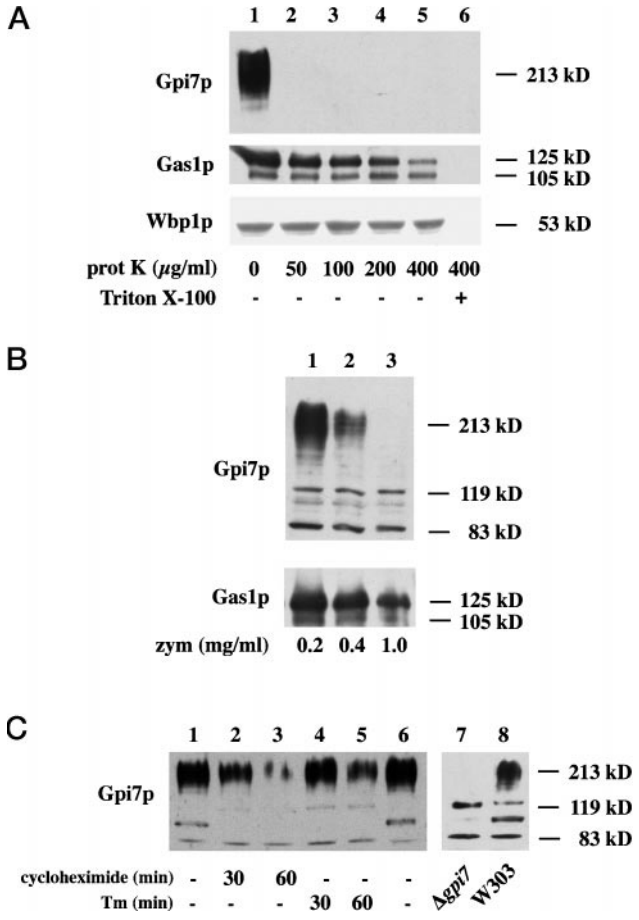

FIG. 9. **Gpi7p is localized at the cell surface.** Cells in the early exponential phase growing at 30 °C in YPD were used. *A*, intact W303 cells were treated with cysteamine chloride and then treated with the indicated concentrations of proteinase K (*prot K*) exactly as described (65) except that 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 Δ*gpi7* cells were either lysed directly or after having been incubated for 30 or 60 min at 10 A_{600}/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.

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. 9*A* low amounts of proteinase K added to intact cells hydrolyzed all of the mature Gpi7p. In contrast, Wbp1p and the 105-kDa ER form of Gas1p were completely resistant to this treatment unless membranes were permeabilized with Triton X-100 (Fig. 9*A, lane 6*). The mature 125-kDa form of Gas1p was found to be partially resistant to proteinase K, indicating either a tighter interaction of Gas1p with some cell wall components or the existence of an internal pool of Gas1p as proposed earlier (50). Crude zymolyase treatment of intact cells also removed all of the mature form of Gpi7p (Fig. 9*B*), whereas recombinant zymolyase left Gpi7p intact (not shown). Longer exposures showed the presence of several minor bands of smaller size all of which were also present in Δ*gpi7* cells except for a 108-kDa form of Gpi7p (Fig. 9*C, lanes 7* and *8*). This material seems to be an ER form in transit to the surface since it was no more detectable if cells were preincubated with either cycloheximide or tunicamycin (Fig. 9*C, lanes 1–6*). These data also indicate that mature Gpi7p is relatively rapidly degraded or becomes resistant to extraction with SDS. Globally these data indicate that the bulk of Gpi7p is exposed at the cell surface but that a small amount of core-glycosylated material is found in the ER in transit to the cell surface. For the moment it is unclear why Gpi7p was completely protected in microsomes, since it has been claimed that PM does not form closed vesicles upon homogenization (51). It is conceivable that Gpi7p resides in special PM subdomains that form closed vesicles upon homogenization or that centrifugation of microsomes generated protease-resistant membrane aggregates (51).

TABLE I
Quantitation of GPI anchor lipids

Cells were labeled with [^{3}H]Ins (5 μCi/A_{600}) for 75 min at 37 °C. Incorporation of [^{3}H]Ins into lipids ranged from 43 to 49% of the radioactivity added to cells. Proteins were extensively delipidated, and anchor peptides were prepared as described (41). Anchor peptides were treated with HNO_2 to liberate the [^{3}H]Ins-P-lipid moieties; the products were desalted by partitioning between butanol and water, and the labeled lipid products were separated by TLC and quantitated by radioscanning. Results of the quantitation of anchor lipids pG1, pC1, and pC2 are given as percentage of the total radioactivity of anchor peptides. The remaining 7–11% that are not accounted for stayed at the origin and represent residual anchor peptides that had not been cleaved by HNO_2 treatment. An aliquot of W303 anchor lipids is shown in Fig. 4*B, lane 11*.

	pG1	pC1	pC2
W303	22.2	63.6	7.4
X2180	24.2	61.2	4.8
gpi8–1	23.8	63.9	2.6
Δ*gpi7*	68.8	19.3	1.1

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 (21). We therefore investigated GPI protein transport in Δ*gpi7*. 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 Δ*gpi7* is specifically retarded. Nevertheless, in rich media Δ*gpi7* cells grow at roughly the same rate as wt cells. They also incorporate [^{3}H]Ins with the same efficiency as wt cells.

The lipid remodeling of GPI anchors is significantly altered in Δ*gpi7*. As seen in Table I, the proportion of ceramides (pC1 and pC2) in anchor peptides from Δ*gpi7* 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 [^{3}H]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 [^{3}H]Ins and [^{3}H]dihydrosphingosine ([^{3}H]DHS) incorporation into GPI proteins. As can be seen in Fig. 10, the ratio of [^{3}H]DHS/[^{3}H]Ins incorporation into proteins is much higher in wt than in Δ*gpi7* (Fig. 10, *lanes 1–4*). The lack of incorporation of [^{3}H]DHS in Δ*gpi7* cannot be explained by an increase of the endogenous production of DHS in Δ*gpi7*, since the difference between wt and Δ*gpi7* persists, even when all endogenous DHS biosynthesis is blocked by myriocin (Fig. 10, *lanes 5* and *6*). The defect in

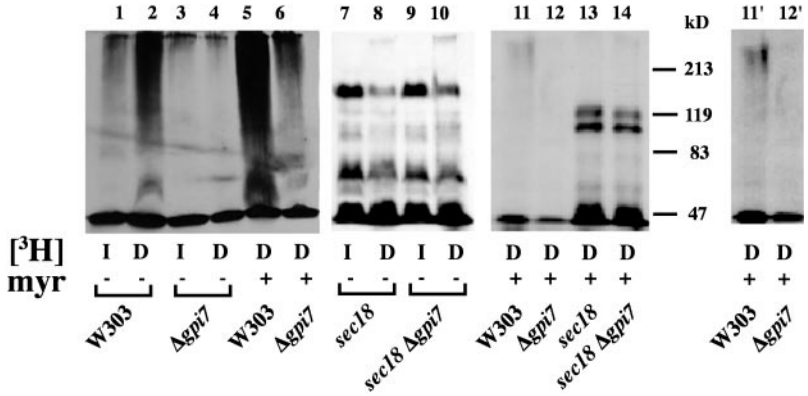

FIG. 10. **Ceramide remodeling of GPI anchors is reduced in Δ*gpi7*.** Cells growing exponentially in SDCUA were used. Cells were labeled by the addition of either 25 μCi of [^{3}H]DHS (*D*) or [^{3}H]Ins (*I*) to an aliquot of 2.5 A_{600} of cells exactly as described (24). *Lanes 1–6*, precultures and labelings were at 30 °C. Cells were preincubated with (+) or without (−) 40 μg/ml of myriocin (*myr*) for 20 min before addition of the radiotracers. *Lanes 7–10*, *sec18* or *sec18* Δ*gpi7* double mutants were precultured at 24 °C and preincubated for 10 min at 37 °C before addition of the tracers. *Lanes 11–14*, cells were labeled in the presence of cycloheximide under conditions in which only the Golgi/PM remodelase (*lanes 11* and *12*) or only the ER remodelase (*lanes 13* and *14*) is probed (Ref. 24, therein Fig. 7 (Golgi/PM remodelase) and Fig. 8 (ER remodelase)). All samples were processed for SDS-PAGE under reducing conditions and fluorography. *Lanes 11′* and *12′* were scanned at an increased sensitivity.

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 [^{3}H]DHS/[^{3}H]Ins incorporation into proteins in the Δ*gpi7*/*sec18* double mutant was the same as in *sec18*. Also, when using stringent conditions under which one observes only ER or only Golgi/PM remodelase (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 Δ*gpi7* (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.[3] 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 in 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 unspecific 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.[3] 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*.

Δ*gpi7* 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.[3] 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 [^{3}H]Ins incorporation into proteins of Δ*gpi7* is not grossly reduced. Thus it is conceivable that some GPI proteins important for cell wall architecture are lacking in Δ*gpi7*. (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

[3] I. Imhof, U. Meyer, A. Benachour, I. Flury, E. Canivenc-Gansel, C. Vionnet, and A. Conzelmann, manuscript in preparation.

TABLE II

GPI7 has homologues in other species

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.c., S. cerevisiae; S.p., Schizosaccharomyces pombe.*

		1	2	3	4	5	6	7	8	9
1	YJL062w = *GPI7, S.c.*									
2	*YA93*, (1175452), *S.p.*	**29**								
3	(1132507), *C. elegans*	**22**	**26**							
4	YLL031c, *S.c.*	22	19	16						
5	(2984587) *homo sapiens*	18	20	16	**24**					
6	(2257562) *S.p.*	20	20	18	**28**	**23**				
7	(2734088) *C. elegans*	19	19	16	**21**	**20**	**23**			
8	*MCD4*, YKL165c *S.c.*	15	15	18	12	12	14	12		
9	(2879870) *S.p.*	16	14	14	12	11	13	1	**39**	

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

Our data further show that ceramide remodeling in the Golgi/PM is significantly reduced in Δ*gpi7,* 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 Δ*gpi7*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/PM remodelase. (iii) We also considered the possibility that Gpi7p itself may be a Golgi/PM remodelase. This latter hypothesis would not directly explain why Δ*gpi7* 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/PM remodeling of GPI proteins in Δ*gpi7* 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 Δ*gpi7* incorporates [^{3}H]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 cell surface. The M4 hypothesis, however, does not explain why M0 and M4 accumulate in Δ*gpi7,* 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 Δ*gpi7.* To save the M4 hypothesis, the accumulation of GPI intermediates in Δ*gpi7* 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. 9*C*). 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

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 H*X*LGXD*XX*GH and DHGM*XXX*G*X*HG. These motifs are also found in two EST clones from human cDNA that have high homology to *MCD4* (NCBI PID 1779747 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 PT*X*T*X*$_8$TG*X*$_2$P which is common to bacterial, viral, plant, and mammalian phosphodiesterases. This homology may suggest that Gpi7p itself is the transferase adding the phosphodiester-linked substituent on Man2. In this context it is interesting to note that the EtN-P on Man3 of the GPI anchor has been shown to be transferred by transesterification using phosphatidylethanolamine as donor of EtN-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 EtN-P.[2] Thus, it is conceivable that not only the *GPI7* subfamily but also other subfamilies are involved in the transfer of EtN-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 GPI structures.

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 hydrophobic membrane protein involved in the addition of EtN-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*/*GPI3*/*CWH6*/*SPT14* (7). Thus, although deficiencies in *PIG-A* are only acquired by somatic cells, deficiencies in the human *GPI7* homologue may be transmittable through the germ line as well.

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Additional results

Construction of a yeast strain expressing a GST-tagged form of Gpi7p

Proteins expressed as fusions with *Schistosoma japonica* glutathione S-transferase (GST) can conveniently be purified using the commercially available glutathione sepharose. With the aim to further characterize Gpi7p, we decided to construct a yeast strain that expresses GST-tagged Gpi7p as the only source of Gpi7p. The GST tag was inserted in a wild-type yeast strain at the *C*-terminus of the genomic copy of *GPI7* by homologous recombination with an insertion cassette containing the GST tag and the selectable marker *KanMX6* as described (Longtine *et al.*, 1998). A clone in which the PCR-made insertion cassette was targeted correctly, as verified by whole yeast cell PCR (Huxley *et al.*, 1990), was labeled with [^{3}H]Ins to investigate if the presence of the GST tag interferes with the function of Gpi7p. As can be seen in Figure 3-1, *Δgpi7* cells accumulate M4, whereas neither wild-type cells nor cells expressing Gpi7p-GST accumulate this GPI intermediate. We therefore conclude that the insertion of the GST tag at the *C*-terminus of Gpi7p does not disturb the function of Gpi7p.

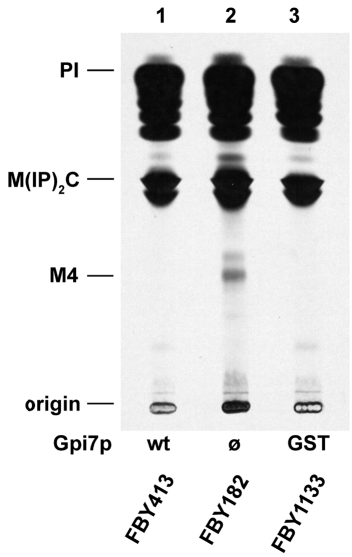

Figure 3-1. Cells expressing Gpi7p with a GST tag at the *C*-terminus have the same lipid profile as wild-type cells.

Exponentially grown cells were labeled with [^{3}H]Inositol (4 μCi/1-2 x 10^7 cells) at 30°C as previously described. Lipids were extracted, desalted, and analyzed by TLC using solvent 1 ($CHCl_3$/CH_3OH/H_2O 10:10:3 v/v/v) and fluorography. FBY413, wild-type; FBY182, *Δgpi7*; FBY1133, *gpi7::GPI7-GST*.

Gpi7p migrates as a 245 kD band in blue native gel electrophoresis

In *Saccharomyces cerevisiae* as well as in mammalian cells, the first step of GPI biosynthesis is carried out by a complex glycosyltransferase consisting of several proteins (Leidich *et al.*, 1995; Schönbächler *et al.*, 1995; Vossen *et al.*, 1995; Leidich and Orlean, 1996; Watanabe *et al.*, 1998; Hong *et al.*, 1999b; Watanabe *et al.*, 2000). The GPI transamidase that transfers the GPI glycolipid onto newly synthesized proteins is also a multiprotein complex and consists of at least three subunits in yeast and four subunits in mammalian cells (Fraering *et al.*, 2001; Ohishi *et al.*, 2001). To investigate if Gpi7p is associated to any proteins under physiological conditions, we prepared digitonin extracts of microsomes and analyzed the proteins by blue native gel electrophoresis. This technique allows the analysis of native membrane protein complexes from biological membranes under conditions that preserve tertiary and quaternary structures of the proteins (Schägger and von Jagow, 1991; Schägger, 1996). Blue native gel electrophoresis has recently been successfully used in our laboratory to investigate the interaction of Gpi8p with other proteins (Fraering *et al.*, 2001). We decided to use cells expressing Gpi7p with a GST tag at its *C*-terminus rather than wild-type cells, since the GST tag allows convenient isolation of Gpi7p (and its potential partners) by affinity chromatography over glutathione-sepharose. The primary translation product of Gpi7p-GST has a predicted molecular weight of 121 kD. Using mouse monoclonal anti-GST antibodies, we detected Gpi7p-GST at a molecular weight of 245 kD, suggesting that Gpi7p-GST is associated with itself or with other protein(s) (Figure 3-2).

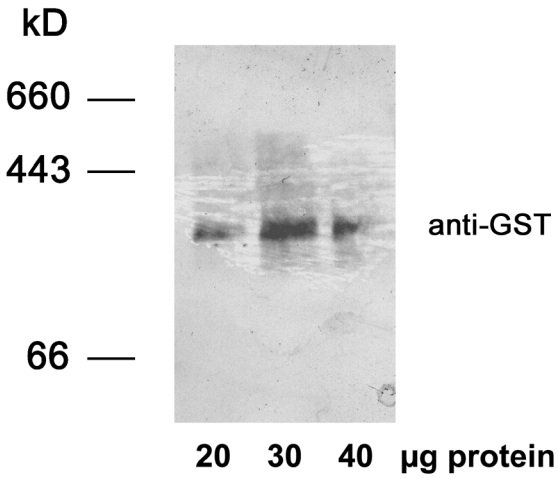

Figure 3-2. Blue native polyacrylamide gel electrophoresis of microsomal membrane proteins.

FBY1133 (*ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 gpi7::GPI7-GST KanMX6*) cells were grown overnight in YPDUA at 30°C. Cells were harvested, microsomes were prepared and membrane proteins solubilized in digitonin in presence of 6-aminocaproic acid. Proteins were processed for blue native polyacrylamide gel electrophoresis. Indicated amounts of proteins were loaded on a 5 – 15% polyacrlyamide gradient gel. Proteins were transferred onto a PVDF membrane and probed with anti-GST antibodies. Marker proteins were bovine serum albumin (monomeric form, 66 kD), apoferritin (443 kD) and thyroglobulin (660 kD).

Affinity purification of Gpi7p-GST and its putative partners

GST-tagged Gpi7p and its putative partner(s) were isolated by affinity chromatography over glutathione sepharose from digitonin extracts of microsomes as described (Fraering *et al.*, 2001). Protein samples were analyzed by SDS-PAGE and silver staining. As can be seen in Figure 3-3, at least four proteins (indicated with a, b, c and d, respectively) were eluted with reduced glutathione.

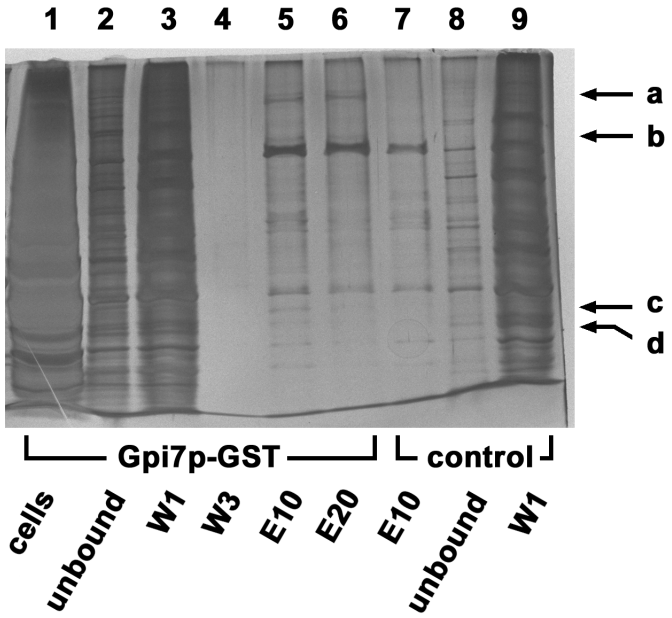

Figure 3-3. Affinity chromatography purification of Gpi7p-GST and its putative partners.

Lysate of FBY1133 (*GPI7-GST*) and FBY413 (wt) cells was incubated with glutathione sepharose 4B. Material bound to the resin was eluted with the use of 10 mM, then 20 mM reduced glutathione (eluates E10 and E20, respectively, derived from 300 OD_{600}). Crude lysate (2.5 OD_{600}), material not bound to the resin (5 OD_{600}) and washes (75 OD_{600}) were loaded on a 6-15% polyacrylamide gel for SDS-PAGE. The gel was stained with silver nitrate. The bands labeled with a, b, c and d correspond to proteins that are absent in the control samples.

Discussion

Using the polyclonal antibody raised against the *N*-terminal hydrophilic part of Gpi7p, we have shown that the bulk of Gpi7p is localized at the cell surface, and that a small amount of Gpi7p is found in the ER (Benachour *et al.*, 1999). The cell surface localization of Gpi7p was unexpected, since the biosynthesis of the GPI anchor is initiated in the ER, and also the transfer of the preassembled GPI to the protein takes place in this organelle. An important issue in this context is to determine whether a) the ER-form of Gpi7p and b) the plasma membrane-bound form of Gpi7p are catalytically active. To date, studies to address this question have not succeeded (Vionnet and Conzelmann, unpublished results). If

surface-localized Gpi7p is the only catalytically active form of this enzyme, this could mean that GPI intermediates cycle between the ER and the plasma membrane before attachment to proteins, or else that the physiological precursor lipid for GPI anchoring is M4.

We have shown that insertion of the relatively large GST tag at the *C*-terminus of Gpi7p does not interfere with the function of Gpi7p, since cells expressing the tagged form as only source of Gpi7p do not accumulate GPI intermediates.

Blue native gel electrophoresis was used to investigate if Gpi7p might be present in a complex under physiological conditions. We prepared microsomes of cells expressing Gpi7p-GST and analyzed solubilized membrane proteins under conditions that preserve tertiary and quaternary structures of protein complexes. The results obtained for Gpi7p-GST were encouraging for further experiments, since using anti-GST antibodies, we could detect a band of 245 kD, suggesting that Gpi7p might stably interact with another protein (Figure 3-2). Indeed, a preliminary affinity purification revealed several bands that specifically co-eluted with Gpi7p-GST (Figure 3-3). These experiments will have to be repeated in order to prepare sufficient amuonts of proteins for analysis by mass spectrometry. Since it has been reported that human GPI7 is associated with PIG-F (Hong and Kinoshita, 2001), it will be interesting to find out if the same is true in yeast.

4. Further characterization of *GPI13*, *MCD4* and *GPI7*

Introduction

Results obtained in several studies (Benachour *et al.*, 1999; Gaynor *et al.*, 1999; Hong *et al.*, 1999a; Flury *et al.*, 2000; Hong *et al.*, 2000; Taron *et al.*, 2000) strongly suggest that the three homologous proteins Mcd4p, Gpi7p and Gpi13p are required for the addition of EthN-P to the first, second and third Man residue of the GPI core, respectively. A hydropathy plot and a sequence alignment of these proteins is shown in Figures 4-1 and 4-2. Consistent with a role for Mcd4p in addition of EthN-P to Man1, we have shown that mutants deficient in *MCD4* accumulate a GPI intermediate that lacks EthN-P on the first Man residue. We have also further characterized cells depleted of Gpi13p and Mcd4p, respectively.

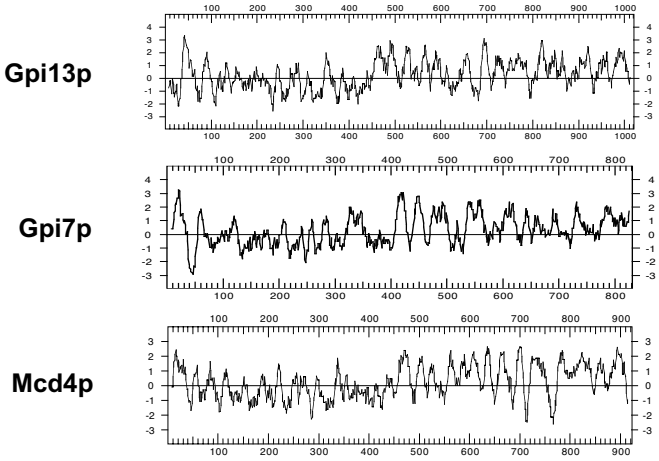

Figure 4-1. Kyte-Doolittle hydropathy plot of Gpi13p, Gpi7p and Mcd4p.
The hydropathy plots were generated with DNA Strider 1.3 program.

Figure 4-2 (next page). Sequence alignment of Gpi13p, Gpi7p and Mcd4p.

Homologies of Gpi13p, Gpi7p and Mcd4p were identified using the ClustalW (version 1.8) program at EBI. Asterisks, colons and periods indicate identity, strong similarity and weak similarity, respectively.

```
GPI13           MDEKTIKKSILSSSNDEKIIYKSRIKKFQKNHKFYIILLVFIAILQFISIAFFTRGFLLS 60
GPI7            ----------------------------MNLKQFTCLSCAQLLAILLFIFAFFPRKIVLT 32
MCD4            ----------------------------MWNKTRTTLLAVGVLFHLFYLWSIFDIYFISP 32
                                             :    :  . :    :   ::*   :: .
GPI13           RHVLDNISSQNETSKLPPRFNKAVILVIDALRFDFAIPVNESHSNYNLNYHNNILSLYDS 120
GPI7            GISKQDPDQDRDLQRDRP-FQKLVFVIIDALRSDFLFDSQISHFNNVHQWLN-------- 83
MCD4            LVHGMSPYQSTPTPPAKR----LFLIVGDGLRADTTFDKVTHPVSGKTEFLAPFIR---- 84
                     .  ..             .::: *.** *  :       .   ::
GPI13           FASDKDASSLLLKFIADPPTTTLQRLKGLTTGSLPTFIDAGSNFDG------TVIEEDNF 174
GPI7            -----TGEAWGYTSFANPPTVTLPRLKSITTGSTPSFIDLLLNVAQD-IDSNDLSEHDSW 137
MCD4            --SLVMNNATYGISHTRMPTESRPGHVAMIAGFYEDVSAVTKGWKSNPVNFDSFFNQSTH 142
                       .:      :  ** :     .: :*    .     .          . :...
GPI13           LKQLHLANKTVKFAG-------DDTWMALFHPFLSNDSFPLESLNVWDLDTVDN-GVMDY 226
GPI7            LQQFIQHNNTIRFMG-------DDTWLKLFPQQWFDFADPTHSFFVSDFTQVDNNVTRNL 190
MCD4            TYSFGSPDILPMFKDGASDPNKVDTWMYDHTFEDFTQSSIELDAFVFRHLDQLFHNSTLN 202
                  .:   :    * .        ***:  .       :    .  *
GPI13           FHDHLQQDKEWDVMIGHMLGIDHVGHKYGPDHFTMREKQIQVDQFID-----WILKSIDD 281
GPI7            PGKLFQEWAQWDVAILHYLGLDHIGHKDGPHSKFMAAKHQEMDSILKSIYDEVLEHEDDD 250
MCD4            STLDYEIRQDGNVFFLHLLGCDTAGHSYRPYSAEYYDNVKYIDDQIPILIDKVNKFFADD 262
                     :   : :* : * ** *  **.  *       :   :*. :           **
GPI13           DTLLVILGDHGMDHTGNHGGDSIDELESTLFLYSKKPDMWRLKETSN-------YNIDNL 334
GPI7            DTLICVLGDHGMNELGNHGGSSAGETSAGLLFLSPKLAQFARPESQVNYTLPINASPDWN 310
MCD4            KTAFIFTADHGMSAFGSHGDGHPNNTRTPLVAWGAGLNKPVHNPFPVSDN--YTENWELS 320
                .* : . .****.  *.**..  .:  : *.  .                  . :
GPI13           GHDYRSVRQIDLVSSLALLMGQPIPFNNLGWPIDEIARNDREWSQFVNSAISQLQLYKDT 394
GPI7            FQYLETVQQIDIVPTIAALFGMPIPMNSVGIIIP--------------------------- 344
MCD4            SIKRNDVKQADIASLMSYLIGVNYPKNSVGELPIAYIDGK-----------ESDKLAALY 369
                    . *:* *:.. :: *:*   * *.:*
GPI13           MQIHHGNDEILEPLAKNISNTPPTSDPEKFVKLGHKYQKVFLQTCEELWAKFDYYSIATG 454
GPI7            ----------------------------DFLQLLPNKLASMKENFMHLWKLSDHHGEVAL 376
MCD4            NNARSILEQYLVKQDEVIDSQFFYKEYFKFVEKSHSHYLEEIETLIQRISEGENYLEQEA 429
                                          .*::   .      :.  .     : :
GPI13           ITLLATSLVLLISITKLIPSIVVNQMVPEFVPGIIIMVLVTNLCFHGIFYVYQQPSFVDQ 514
GPI7            DDFTAEDIYTKMYTIQETLTKSATNYNYPLLTLAFVGFLIITIIAIYVLLRYSGPDFWQL 436
MCD4            ITLTEELMQITLEGLHYLTTYNWRFIRTIVTFGFVGWIFFSFIIFLKSFILENVIDDQKA 489
                  :    :   :   :   :         .    .  .:.  :     :   .  .  .
GPI13           FWGTLLATAIGIIIGCYITIFDRYNFIWIAMRLGETLADYWSRIAVMFMIIHALLFTSNS 574
GPI7            RVSSLSVLLVSIILG--VSTFASS--FIEEEHQ------LWWWIVTAFSAVPLFVYRLN- 485
MCD4            SPLSHAVFGSIGILLNWILFYQHSPFNFYMYLLFP--LYFWSYIFTNRSVLRSGIKEFFK 547
                   :  .     *:   :  :                    *  * .    :   :
GPI13           FTIWEDRIVAFLLSTFGMLTLYEFVFLPKRQSTTALLTATISEKEGTTSGVNPSTANSNY 634
GPI7            --------VLIIVRWFIMMACVRSIKFWNNSGQKFIYSNVMSNLLNQNPSWKWCLNMLTF 537
MCD4            G----TSPWKRVLITISIISVYEGIVYGFFHRWTFTLITNILAFYPFICGVRELSVNILW 603
                          ::  : :::  . :        .      :        . .       :
GPI13           LPLTRFARLLGGYHSAVLIIFTRLASMITICREEQGEYCIPTFNNQNNSSWWVLGLCFLM 694
GPI7            LVLIMASAGFQVLHFIVTTILV--------------------------------GLCFTY 565
MCD4            IITSVLLSTFTLFDAVKIEDLNQIHLAG-------------------------LLIILS 637
                :        :   .      :                                  * :
GPI13           IFILPACITGYYNLTSSYQAAAPIWINVFLKGILGLNFVYWSLTSLENNSAVIAIPFLRD 754
GPI7            KIS--WEIVNGN------QAEIPLFMHDLLAKID---FAPT----ES-NLIVLARVFFQA 609
MCD4            AFYALYKIHSRIN-----SYTRAIFAIQISLVAAMLAVTHRSVISLQLRQGLPRESQVAG 692
                 :     * .         .   .::   :         ..       . .  :     .
GPI13           VTIFKFTLARIIAGFSLIASNVGWLMGPLCIKLNIHNTDVKSHEATILGYTNIYGSEFFL 814
GPI7            WAIVVISRLVLTKLKVLNKN------------Y--LIKDMKVYITILLMFQTSSQNIGQF 655
MCD4            WIIFFVSLFVMPILHYRKPNN---D------YKVRLLIIYLTFAPSFIILTISFESLFYF 743
                  *. .:   :        .                        . . ::       .   :
GPI13           LVINVLISILLFNKPLAQLSYFLMCNQLLSILEIIDLLKLKENIIGPIALGLLSYQHFFT 874
GPI7            LVFQILESQIFY---------------FFQNIPTASLTSTSKIYFSNLVSLILQNFTFFQ 700
MCD4            LFTSYMVQWIEIEN-------------KIKEMKTQKDENWLQVLRVSVIGFFLLQVAFFG 790
                *. . : . :                  :. :   .  .  :     :   :*    **
GPI13           TGHQATIPSVQWDIGFM-LSEKVTFPFTQIAIILNTFGPHILVSLSVALLTLWSQPPDVL 933
GPI7            FGGTNSISTIDLGNAYHGVSSDYNIYVVGILMSVANFAPAIYWSMLPWSINYASIP---- 756
MCD4            TGNVASISSFSLESVCR-LLPIFDPFLMGALLMLKLIIPYGLLSTCLGILNLKLN----- 844
                 *   :*.:..       :       .    : :  : *    *     :.
GPI13           KPQTLLGRIVSNCGILLTYNTILCLSSFIWVTHFRRHLMVWKIFCPRFIFASLSLIVTQL 993
GPI7            -AQVKLQTFIRSKLPAFTYHCIFGTCLMTACVVLRFHLFIWSVFSPKLCYFLGWNFVMGL 815
MCD4            FKDYTISSLIISMSDILSLNFFYLLRTEGSWLDIGITISNYCLAILSSLFMLILEVLGHV 904
                  :  :  :: .    :: : :           :   :  : :      :     .:  :
GPI13           VVTFGTIAFASGRLIKHINDIFWK 1017
GPI7            LNGWLPELALLCALD--------- 830
MCD4            LLKNVIIQDKTKKTQ--------- 919
                :
```

Results

Mutation or depletion of *MCD4* lead to accumulation of GPI lipids

Saccharomyces cerevisiae mcd4-174 mutants are deficient in inositol incorporation into proteins and accumulate several [^{3}H]inositol-labeled GPI precursors (Gaynor *et al*., 1999). We wanted to reproduce the abnormal lipid profile found in *mcd4-174* mutants. We have previously observed that Mcd4p-depleted cells accumulate only very low amounts of GPI intermediates when labeled with [^{3}H]inositol (Flury *et al.*, 2000), consistent with results published by Gaynor *et al*. (1999). We therefore decided to analyze the *in vitro* GPI biosynthesis in yeast strains carrying either a temperature-sensitive mutation in *MCD4/SSU21* (Packeiser *et al.*, 1999) or the wild-type *MCD4* gene under control of a galactose-inducible promoter (Flury *et al.*, 2000). Microsomes from a wild-type strain make the complete GPI precursor CP2 irrespective of the carbon source on which cells have been grown (Figure 4-3, *lanes 1 and 2*). In contrast, *MCD4*-depleted microsomes accumulate a GPI intermediate termed lipid 4c (Figure 4-3, *lane 4*). It should however be noted that these *MCD4*-depleted microsomes are able to make CP2, although considerably less than wild-type. When microsomes derived from a strain carrying a temperature-sensitive mutation in *MCD4* are labeled at the restrictive temperature, a stronger block of GPI biosynthesis is observed (Figure 4-3, *lane 5*), and neither M4 nor CP2 is made. Lipid 4-c is less polar than lipid 031b, suggesting that it is an earlier intermediate in GPI biosynthesis.

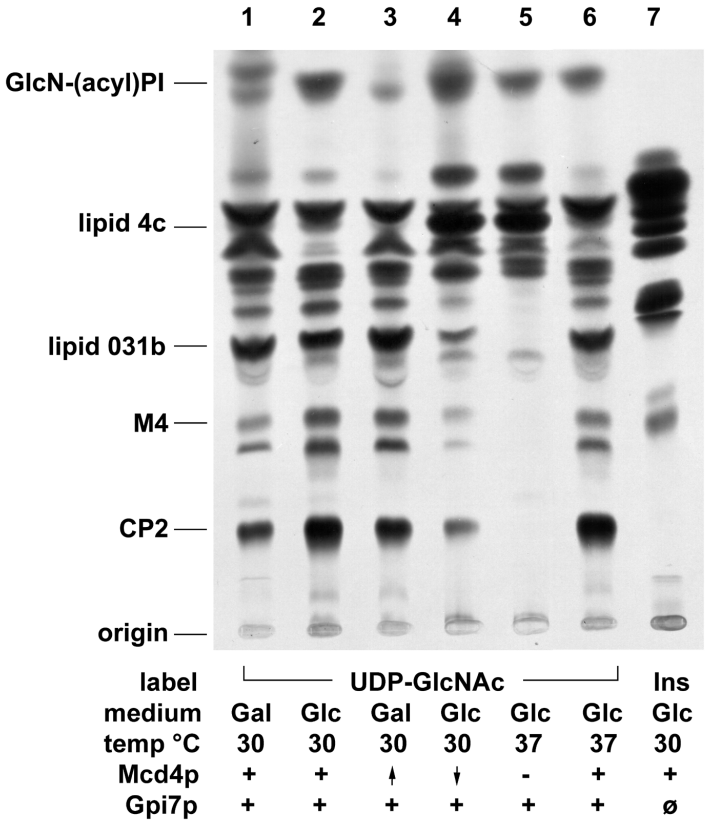

Figure 4-3. Cells deficient in *MCD4* accumulate GPI intermediates *in vitro*.

Lanes 1-6, microsomes were prepared as previously described (Flury *et al.*, 2000), except that all incubations were carried out at the temperatures indicated above. Cells in *lanes 5 and 6* were preincubated for 2 h at 37 °C before conversion to spheroplasts. Microsomes were incubated with 6 μCi of UDP-[^{3}H]GlcNac, GDP-Man, tunicamycin, CoA and ATP at the indicated temperatures for 1 h. *Lane 8*, cells were labeled with [^{3}H]inositol (Ins) and processed as previously described (Flury *et al.*, 2000). *Lanes 1 and 2,* FBY413 (wild-type); *lanes 3 and 4*, FBY1104 (*pGAL1,10-MCD4*); *lane 5*, 521-17A-H42 (*ssu21/mcd4*^{ts}); *lane 6*, 17A-H42 (wild-type); *lane 7*, FBY182 *(Δgpi7*). Lipids were extracted, desalted, and analyzed by TLC using solvent 1 ($CHCl_3$/CH_3OH/H_2O 10:10:3 v/v/v) and fluorography.

Head group analysis of a GPI intermediate accumulating in cells depleted for *MCD4*

Mouse embryonal carcinoma cells disrupted for the *MCD4*-homologue *PIG-N* accumulate several GPI intermediates, the two most polar probably having 3 and 4 mannoses respectively and EthN-P both on Man2 and Man3, but not on Man1 (Hong *et al.*, 1999a). Together with the finding that the surface expression of Thy-1 was only partially affected in *Pig-n* knockout cells, these results indicate that in mammalian cells, EthN-P on Man1 is not essential for attachment of GPI anchors. To date, there are no reports on the structures of GPI lipids that accumulate in *MCD4*-deficient *Saccharomyces cerevisiae* strains. The presumed function of Mcd4p is the addition of EthN-P on the first mannose of the GPI core. If this is true, we would expect that GPI lipids in *mcd4* mutants lack this modification. We therefore wanted to analyze the structure of the major lipid accumulating in a *mcd4* mutant. As shown in Figure 4-3, the mutation present in the strain 521-17A-H42 leads to the absence of the complete GPI precursor CP2 and accumulation of a GPI intermediate termed lipid 4-c. For structural characterization of lipid 4-c, microsomes from strain 521-17A-H42 were labeled with UDP-[^{3}H]GlcNAc and lipid 4-c was purified by preparative TLC. Analysis of the hydrophilic head group indicates that lipid 4-c contains a Man_2-GlcN-Ins core structure and that both mannoses can be removed by treatment with jack bean α-mannosidase (JBAM) (Figure 4-4).

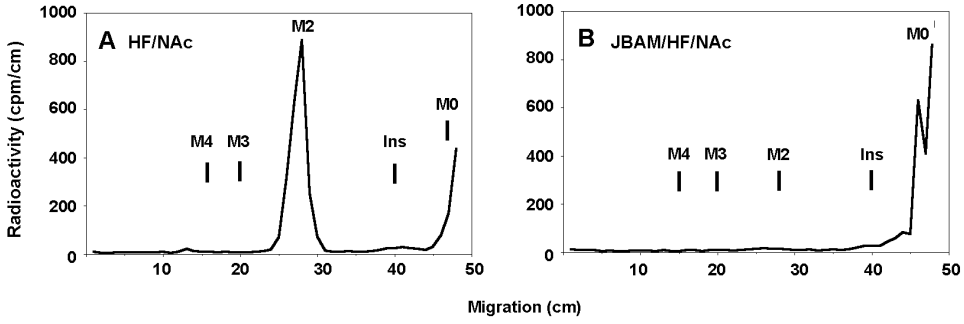

Figure 4-4. Head group analysis of lipid 4-c.

Soluble headgroups were obtained by treatment of purified glycolipids with monomethylamine:water 10:3 for 1 h at 53 °C (Guillas *et al.*, 2001). Non-hydrolyzed lipids were removed by butanol extraction. The water-soluble headgroups were treated as indicated above. The generated fragments were analyzed by paper chromatography in methylethylketone/pyridine/water (20:12:11) and compared to radiolabeled chromatography standards as previously described (Flury *et al.*, 2000).

Assay for multicopy suppression

The three putative ethanolamine phosphate transferases Gpi13p, Gpi7p and Mcd4p show significant homology to each other in the *N*-terminal, hydrophilic region (Figure 4-2). Gpi13p and Mcd4p are essential proteins, but Gpi7p is dispensable for yeast viability (Benachour *et al*., 1999; Gaynor *et al*., 1999; Zhang *et al*., 1999; Flury *et al*., 2000). We wanted to investigate if overexpression of these three genes from a *2μ* plasmid could overcome the growth defect of cells depleted for Gpi13p or Mcd4p. For this experiment, we transformed yeast strains having *GPI13* or *MCD4* under control of the repressible *GAL1,10* promoter as well as a wild-type strain with 2*μ*-based plasmids which contained the gene of interest controlled by its own promoter. The growth of plasmid-bearing clones was monitored on glucose-containing plates with or without the cell wall perturbing drug calcofluor white (CFW). Wild-type cells transformed with either an empty, *URA3*-based *2μ*-vector (ø) or *2μ*-vectors encoding *MCD4*, *GPI7* and *GPI13* respectively, were able to grow at all dilutions in the presence or absence of CFW (Figure 4-5C). In contrast, both Gpi13p- and Mcd4p-depleted cells transformed with the same empty vector showed a reduced growth rate on plates lacking CFW, confirming that both Gpi13p and Mcd4p are required for viability. In the presence of 100 *μ*g/ml CFW, the growth inhibition of the depletant cells was significantly enhanced. As can be seen in Figure 4-5A, the growth phenotype of Gpi13p-depleted cells on both types of plates could only be rescued by overexpression of *GPI13*, but not by overexpression of *MCD4* and *GPI7*. In the same way, cells depleted for Mcd4p could not be rescued by overexpression of *GPI7* and *GPI13*, but growth was restored when *MCD4* was present on a *2μ* plasmid (Figure 4-5B). However, Gpi13p- and Mcd4p-depleted cells "rescued" by the homologous plasmid grew slightly less on CFW-containing plates than on plates lacking the drug.

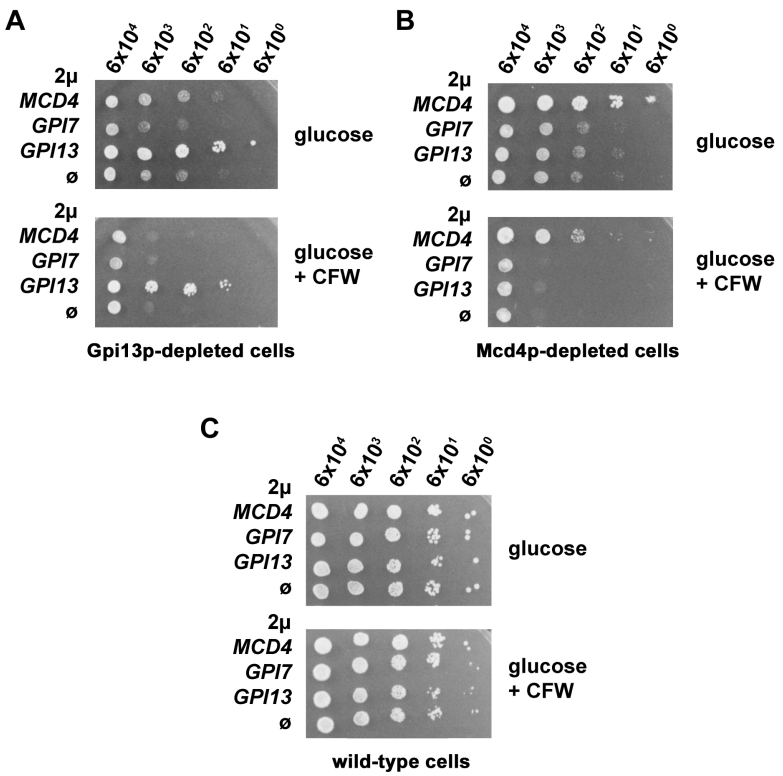

Figure 4-5. Growth test of cells depleted for Gpi13p and Mcd4p.

A) Gpi13p-depleted cells, B) Mcd4p-depleted cells, and C), wild-type cells were transformed with *2µ* plasmids containing *MCD4*, *GPI7*, *GPI13* ORFs or empty vector (ø), respectively. The indicated number of cells was spotted onto agar plates with or without 100 µg/ml CFW. Plates were photographed after 3 days at 30 °C.

Depletion of Gpi13p and Mcd4p leads to induction of the Unfolded Protein Response (UPR) signaling cascade

Accumulation of misfolded or improperly assembled proteins in the ER leads to induction of the unfolded protein response (UPR) signaling cascade. The UPR pathway is the subject of numerous reviews (Shamu, 1997; Chapman *et al.*, 1998; Shamu, 1998; Hampton, 2000; Patil and Walter, 2001). The level of unfolded proteins in the ER is increased in different situations, e.g. reduced protein glycosylation, increased production of misfolded mutant proteins, or altered redox status of the ER lumen. In response to UPR, cells increase the transcription of ER-resident chaperones such as Kar2p (BiP) and protein disulfide isomerase (PDI). The UPR-element (UPRE) is a 22-bp sequence found in the promoters of UPR target genes, and it is both necessary and sufficient for transcriptional activation by the UPR. A convenient reporter system to monitor activation of the UPR is based on

UPRE. A *lacZ* reporter gene is fused to an artificial promoter containing UPRE in the plasmid pUPRE (Menzel *et al.*, 1997). Induction of UPR and thus expression of β-galactosidase can be monitored on agar plates containing the chromogenic substrate X-Gal. It has been reported that inhibition of protein glycosylation by tunicamycin disrupts protein folding and thus activates UPR. We wanted to test if inhibition of GPI anchoring had the same effect. We transformed cells with the pUPRE reporter plasmid, depleted Gpi13p and Mcd4p respectively by shifting cells onto glucose-containing plates and monitored the appearance of blue color. As can be seen in Figure 4-6C, wild-type cells remain white, whereas both Gpi13p- and Mcd4p-depleted cells turn blue on plates containing X-Gal. This result indicates that inhibition of GPI anchoring also leads to activation of the UPR signaling cascade.

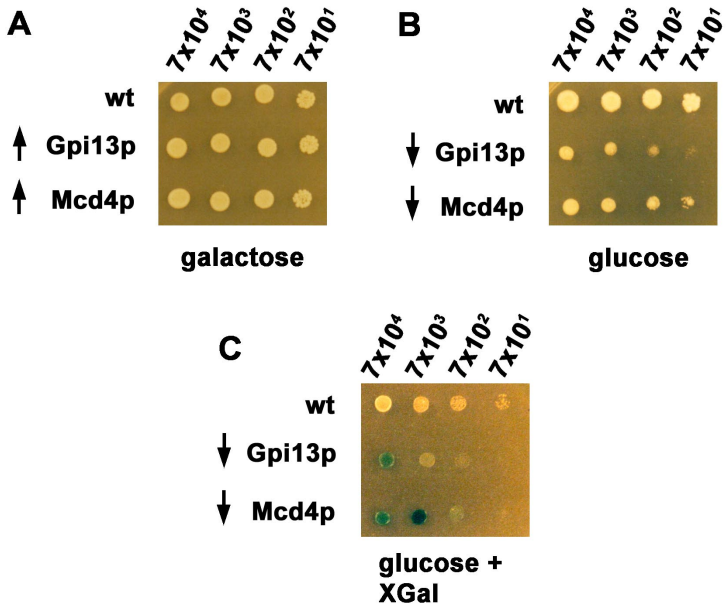

Figure 4-6. Depletion of Gpi13p or Mcd4p lead to induction of the unfolded protein response.

FBY413 (wt), FBY1102 (*pGAL1,10-GPI13*) and FBY1104 (*pGAL1,10-MCD4*) cells were transformed with pUPRE. The indicated number of exponentially grown cells were spotted onto agar plates containing A) galactose; B) glucose; and C) glucose and X-Gal. Plates were photographed after 4d at 30 °C. ↑, overexpression; ↓, depletion.

Discussion

Mcd4p was previously shown to be an essential component of the GPI biosynthetic pathway in *Saccharomyces cerevisiae* (Gaynor *et al.*, 1999). In contrast, the mammalian *MCD4* homologue *PIG-N* is dispensable for viability, since *Pig-n* knockout mice were viable and only partially affected in the surface expression of the GPI-anchored protein Thy-1 (Hong *et al.*, 1999a). GPI precursors in *Pig-n* knockout mice were not modified with EthN-P on Man1, suggesting that PIG-N is involved in the addition of this side chain (Hong *et al.*, 1999a). We have characterized the major lipid accumulating in a *mcd4* mutant in order to demonstrate that yeast Mcd4p is also involved in the transfer of EthN-P to Man1. The *mcd4-174* mutant accumulated trace amounts of several [^{3}H]Ins-labeled GPI lipids (Gaynor *et al.*, 1999), and the same was found when Mcd4p was depleted using the repressible *GAL1,10* promoter (Flury *et al.*, 2000). We therefore decided to analyze *in vitro* GPI biosynthesis in Mcd4p-depleted cells as well as in the temperature-sensitive *mcd4/ssu21* mutant (Packeiser *et al.*, 1999). Whereas GPI biosynthesis was not strongly inhibited in Mcd4p-depleted cells, *mcd4/ssu21* mutants were unable to synthesize the complete GPI precursor CP2 and accumulated predominantly a lipid denoted lipid 4-c. Head group analysis of lipid 4-c showed that it contained two mannose residues and lacked EthN-P on Man1. This result was consistent with reports from studies with the fungal product YW3548, which was shown to specifically inhibit EthN-P addition to Man1 (Sütterlin *et al.*, 1997b; Sütterlin *et al.*, 1998; Hong *et al.*, 1999a). Indeed, it was shown that yeast cells treated with YW3548 also accumulated Man_2-GlcN-acylPI (Sütterlin *et al.*, 1997b). Comparison of results obtained in yeast and mammalian cells revealed differences in the GPI biosynthetic pathway in these organisms. In mammalian *in vitro* systems, YW3548 inhibited the generation of (EthN-P)Man-GlcN-acylPI in the GPI biosynthetic pathway I (see Figure 4-7 for a scheme of mammalian GPI biosynthesis), but GPI intermediates containing more than one mannose and lacking EthN-P on Man1 could nevertheless be formed in pathway II (Hong *et al.*, 1999a). It seems that when EthN-P is added to Man1 In mammalian cells, this addition occurs prior to addition of the second mannose residue (Hirose *et al.*, 1992). In *Saccharomyces cerevisiae*, EthN-P is likely to be added to Man_2-GlcN-acylPI, since YW3548-treated wild-type cells as well as *mcd4/ssu21* mutants accumulate this intermediate. Yeast Gpi10p, which is responsible for the addition of the third mannose residue, is unable to use Man_2-GlcN-acylPI efficiently as a substrate, since none of the GPI intermediates synthesized by *mcd4^{ts}* microsomes contained more than two mannoses (our unpublished observation). In contrast, PIG-B, the mammalian Man3

transferase, seems to be able to use the Man_2-GlcN-acylPI made in pathway II as a substrate, since Man_3-containing GPIs were formed by cells blocked with YW3548 (Hong *et al.*, 1999a).

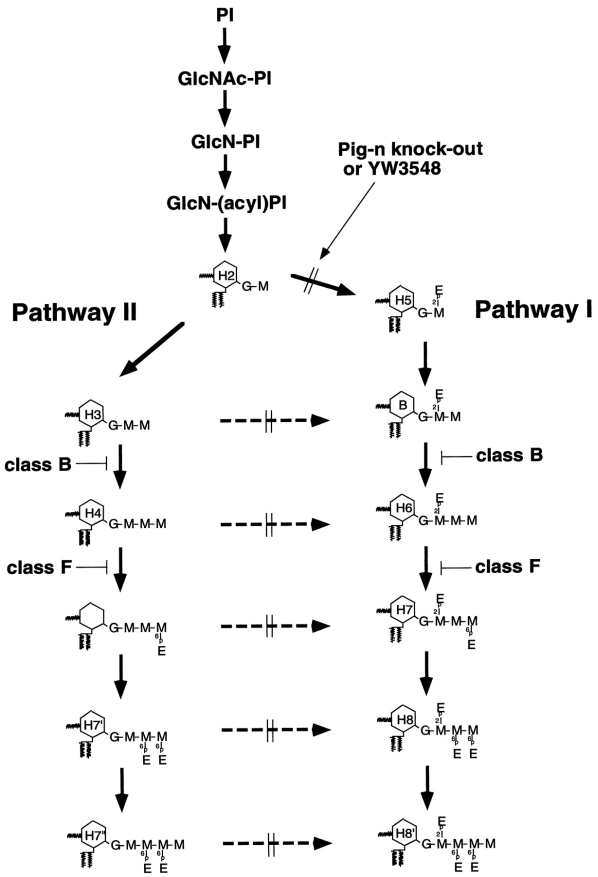

Figure 4-7. A branched pathway for GPI biosynthesis in mammalian cells.
From Hong et al., 1999.

Mammalian as well as yeast results are consistent with a role for Mcd4p in transferring EthN-P to Man1. However, there is recent evidence that not all alleles of *mcd4* show defects in GPI anchoring, suggesting that Mcd4p might have an additional function. The *mcd4-P301L* mutant was identified in a screen designed to isolate yeast strains deficient in the transport or metabolism of PS and PE (Storey *et al.*, 2001). In a *psd1Δ* background, mutation of proline 301 to leucine in Mcd4p caused temperature-sensitivity in absence of exogenous ethanolamine or choline. The growth of the mutant could be improved by the presence of *PSD2* on multicopy plasmid, suggesting that the activity of Psd2p is somehow altered by the *mcd4-P301L* mutation. Both *mcd4-174 psd1Δ* and *mcd4-P301L psd1Δ* mutants were ethanolamine auxotrophs, suggesting that ethanolamine requirement is

common to *mcd4* alleles, and that Mcd4p might be important for the function of Psd2p. But interestingly, unlike the *mcd4-174* mutation, the novel mutation in *MCD4*, *mcd4-P301L*, did not affect [^{3}H]Ins incorporation into proteins and did not lead to accumulation of GPI intermediates, and consistent with this, *mcd4-P301L* cells had normal cell wall maintenance. Neither *mcd4-P301L* nor *mcd4-174* cells were defective in PE synthesis. However, different reasons could be responsible for the observed ethanolamine auxotrophy in the mutant cells. In *mcd4-174* cells, ethanolamine auxotrophy might be due to accelerated degradation of PE to free phosphoethanolamine, since increased amounts of the latter were detected. In contrast, in *mcd4-P301L* cells, PE turnover was normal, indicating that the decreased PE pool observed in these cells was not due to PE degradation, and pinpointing the lesion in this mutant to a process occurring between PS synthesis and decarboxylation.

In view of the high homologies of Gpi7p, Gpi13p and Mcd4p, we investigated if overexpression of one of these genes from a 2*μ*-vector could rescue cells deficient in another gene (Figure 4-4). The observation that growth could only be restored when the depleted gene was present on a plasmid, was not suprising. Indeed, a similar experiment performed in mammalian *Pig-o* knockout cells showed that in contrast to *Pig-o* cDNA, expression of *Pig-n* cDNA was not able to restore surface expression of Thy-1 (Hong *et al.*, 2000). We also performed the multicopy suppressor experiment with *Δgpi7* cells, and we could confirm that *Δgpi7* is hypersensitive to CFW (results not shown). However, none of the plasmids was able to restore growth of *Δgpi7* cells on CFW-containing plates, not even the plasmids pBF41 (2*μ*, *GPI7*) and pBF43 (CEN, *GPI7*) containing the *GPI7* ORF. This was unexpected, since both plasmids were shown to suppress the accumulation of the M4-glycolipid in *Δgpi7* cells (Benachour *et al.*, 1999). However, suppression of the growth defect on CFW-containing plates by pBF41 or pBF43 had not been tested at that time. We will repeat the multicopy suppression experiment with the original *gpi7* mutants (e.g. *gpi7-1*) instead of *Δgpi7* cells (Benghezal *et al.*, 1995; Benachour *et al.*, 1999).

Cellular survival of ER stress such as the accumulation of unfolded proteins requires the unfolded protein response (UPR), a stress response present in all eukaryotes studied to date. As a result, the cell induces transcription of genes encoding proteins that help correct folding, such as Kar2p (BiP) and PDI (protein disulfide isomerase). However, chaperones such as Kar2p are by far not the only proteins regulated by UPR. In yeast, whole-genome expression profiling using DNA-microarrays has shown that the UPR regulates more than

350 genes, which is 5% of the genome (Travers *et al.*, 2000). An important number of these UPR-regulated genes play a role in different aspects of ER function, such as protein translocation, folding, glycosylation, GPI anchoring, ER-associated protein degradation (ERAD), and lipid metabolism. A recent genetic screen for mutants that are dependent on UPR activation for viability also revealed several classes of mutants deficient in the secretory pathway (Ng *et al.*, 2000). Cells disrupted for *IRE1* are viable under normal conditions, but not under ER stress. Mutations that were synthetically lethal when combined with *Δire1* comprise the GPI biosynthetic genes *GPI10* and *MCD4* (Ng *et al.*, 2000). *MCD4* was also found to be regulated by the UPR in the study by Travers *et al.* (2000), where also *GAA1*, *GPI12* and *GPI7*, were identified as targets of the UPR. Therefore, our observations that depletion of Gpi13p and Mcd4p induced the UPR is not surprising. Both depletion of Gpi13p and Mcd4p lead to a reduction of inositol incorporation into proteins, suggesting that proteins that do not receive a GPI anchor are somehow sensed as being unfolded. We speculate that the UPR would also be induced in other mutants deficient in GPI anchor biosynthesis or attachment. Mutant forms of Gas1p that have an unacceptable GPI anchor attachment site were shown to accumulate in the ER (Nuoffer *et al.*, 1993). We would also expect an induction of the UPR in this case, since the cells somehow have to cope with unanchored Gas1p.

5. Conclusion

The modification of mammalian GPI anchors with EthN-P side chains on Man1 and Man2 had been known for several years, when similar side chain modifications were first reported from the yeast *Saccharomyces cerevisiae* (Canivenc-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998; Benachour *et al.*, 1999). The presence of EthN-P on Man1 on GPI precursors had first been revealed in mutants deficient in the transfer of the third Man residue to the GPI core (Canivenc-Gansel *et al.*, 1998; Sütterlin *et al.*, 1998). Evidence for a further phosphodiester-linked substituent on Man2 came from studies with yeast *gpi7* mutants. In comparison to the complete GPI precursor glycolipid CP2 which accumulates in transamidase mutants, the most polar GPI accumulating in *gpi7* cells lacked a HF-sensitive side chain (possibly EthN-P) on Man2, suggesting that the corresponding protein, Gpi7p, might be involved in the addition of a side-chain to Man2 of the GPI core (Benachour *et al.*, 1999). The *GPI7* gene was cloned by complementation of a *gpi7-1 gpi8-1* double mutant. Homology searches have shown that two ORFs of *Saccharomyces cerevisiae* encoded proteins closely related to Gpi7p, *YKL165w/MCD4* and *YLL031c/GPI13*. It was therefore tempting to speculate that the proteins encoded by *YKL165w/MCD4* and *YLL031c/GPI13* might play a role in the addition of EthN-P to Man1 and Man3 of the GPI core. The aim of this thesis was the identification and characterization of EthN-P transferases. Unlike *GPI7*, both *YKL165w/MCD4* and *YLL031c/GPI13* were reported to be essential for viability. To find out if Mcd4p and Gpi13p play a role in GPI anchor biosynthesis, we placed both genes under control of the repressible *GAL1,10* promoter and studied the phenotype of cells depleted of either *MCD4* or *GPI13*. We first investigated if cells depleted of Mcd4p or Gpi13p accumulated [^{3}H]Ins-labeled GPI intermediates, a feature of cells deficient in GPI biosynthesis or transfer to proteins. Whereas Mcd4p-depleted cells only accumulated minor amounts of abnormal [^{3}H]Ins-labeled lipids, Gpi13p-depleted cells accumulated two abnormal lipids in sufficient amount for characterization. We and others have shown that cells depleted of Gpi13p accumulate GPI intermediates lacking EthN-P on Man3, suggesting that this protein might be involved in EthN-P transfer to Man3 (Flury *et al.*, 2000; Taron *et al.*, 2000). Mcd4p was also shown to be required for GPI anchor biosynthesis, although the step defective in *mcd4-174* mutants had not been pinpointed (Gaynor *et al.*, 1999). Analysis of GPI precursors in mammalian cells disrupted for the *MCD4*-homologue *Pig-n* had revealed that these GPIs lacked EthN-P on Man1, suggesting that Mcd4p/PIG-N are required for addition of EthN-P to Man1 (Hong *et al.*, 1999a). During

this thesis work, we have shown that GPI precursors from yeast *mcd4*^{ts} mutants also lack EthN-P on Man1.

The subcellular localization of members of the *GPI7/MCD4/GPI13* family has recently been investigated in yeast and in mammalian cells (Benachour *et al.*, 1999; Gaynor *et al.*, 1999; Hong *et al.*, 1999a; Flury *et al.*, 2000; Hong *et al.*, 2000). Yeast *MCD4* contains a KKXX ER-retention/retrieval sequence, whereas *GPI7* and *GPI13* lack this signal. Indeed, Mcd4p is localized in the ER (Gaynor *et al.*, 1999). A *myc*-tagged form of Gpi13p was also shown to be localized in the ER (Flury *et al.*, 2000). ER localization was also found for a GST-tagged form of PIG-O, the mammalian homologue of Gpi13p (Hong *et al.*, 2000). Interestingly, yeast strains in which the ER retention signal of Mcd4p is mutated grew like wild-type cells, suggesting that this signal is not required for normal function and localization of Mcd4p (Gaynor *et al.*, 1999). Gpi13p might be retained in the ER *via* an interaction with another protein. Indeed it was shown in mammalian cells that the Gpi13p-homologue PIG-O is stabilized in the ER because of its interaction with PIG-F (Hong *et al.*, 2000). PIG-F seems also to be required for the stabilization of human Gpi7p in the ER (Hong and Kinoshita, 2001). In our studies, the bulk of Gpi7p was shown to be localized on the plasma membrane, and only a small amount was found in the ER. In view of the unexpected localization of yeast Gpi7p, future experiments include the production of new anti-Gpi7p antibodies in order verify the subcellular localization of this protein.

The postulated function of Gpi13p, Gpi7p and Mcd4p, the transfer of EthN-P to GPI anchors, has however not yet been confirmed in *in vitro* activity tests with purified enzymes and putative substrates. Experiments pointing in this direction will surely have to be performed in the future to show that Gpi13p, Gpi7p and Mcd4p indeed are EthN-P transferases. The reported homology of Gpi13p and family members with phosphodiesterases and pyrophosphatases suggested that the conserved domains might be important for the function of these enzymes. Indeed, the mutation in *mcd4-174* maps to an amino acid not only highly conserved in the mammalian and *S. pombe* homologues of Mcd4p, but also in its yeast homologues and in the known phosphodiesterases and pyrophosphatases (Gaynor *et al.*, 1999). Further indication that the conserved regions are important for the function came from studies with Gpi7p (Egger, 2000). Amino acids 50 to 192 or 192 to 321 of the Gpi7p protein sequence were deleted, and the resulting truncated forms of Gpi7p were expressed in *Δgpi7* cells from either 2*μ* or CEN-based vectors. The presence of either plasmid did not suppress the accumulation of the M4 glycolipid in the

Δgpi7 background, indicating that the conserved regions are important for proper function of Gpi7p.

If Gpi7p is the transferase which adds a HF-sensitive side chain, possibly EthN-P, onto Man2, it will be intresting to elucidate the physiological function of this side chain. The fact that Gpi7p is not an essential protein means that either another protein can functionally replace it in some cases, or that the side-chain transferred by Gpi7p is not required for growth under certain conditions. However, even though Gpi7p is dispensable for growth under normal conditions, there is no doubt that Gpi7p-deficient cells have a problem with GPI-anchoring (Benghezal *et al.*, 1995; Benachour *et al.*, 1999; Flury *et al.*, 2000). Possible functions of the side chain attached by Gpi7p were discussed (Benachour *et al.*, 1999). Interestingly, the reported temperature-sensitivity of a *Δgpi7* strain could be suppressed by 1 M sorbitol (Tohe and Oguchi, 1998), suggesting that its failure to grow at 37 °C was due to cell wall defects as a consequence of the lack of Gpi7p.

Despite the high similarity of yeast and mammalian proteins involved in EthN-P transfer, several differences seem to exist, as it is the case for the transfer of the bridging EthN-P to Man3. Yeast cells depleted of Gpi13p are unable to add EthN-P onto Man3, whereas mammalian *Pig-o* knockout cells are. Both Gpi11p-deficient yeast cells and mammalian *PIG-F* mutant cell lines were unable to express GPI-anchored proteins. However, one of the two GPI precursors accumulating in Gpi11p-depleted yeast cells contained EthN-P on Man3 (and was therefore a potential substrate for the transamidase) (Taron *et al.*, 2000), whereas GPI intermediates from mammalian *PIG-F* mutant cell lines lacked EthN-P on Man3 (Sugiyama *et al.*, 1991; Puoti and Conzelmann, 1993). Therefore, results obtained in mammalian cells suggested that PIG-F rather than PIG-O was required for the addition of EthN-P to Man3, whereas in yeast, Gpi13p was a better candidate for the EthN-P transferase than Gpi11p. The finding that PIG-F played a role in the stabilization of both PIG-O and GPI7 (Hong and Kinoshita, 2001) might be an indication that PIG-F and PIG-O, but also PIG-F and GPI7 function in concert in mammalian cells. The same could be true in yeast. This implicates a role for PIG-F/Gpi11p in the transfer of EthN-P onto both Man2 and Man3. Therefore, the observed differences in yeast and mammalian cells might be explained by different extents of dependence on partnership with Gpi11p/PIG-F (discussed in Taron *et al.*, 2000). In yeast, transfer of EthN-P to Man3 is independent of Gpi11p, whereas in mammalian cells, PIG-F plays a major role in this reaction step.

A branched pathway has been proposed for GPI anchor biosynthesis in mammalian cells (Figure 4-7). Based on the characterization of GPI intermediates from different yeast mutants, a branched pathway was also proposed for GPI biosynthesis in *Saccharomyces cerevisiae* (Figure 5-1).

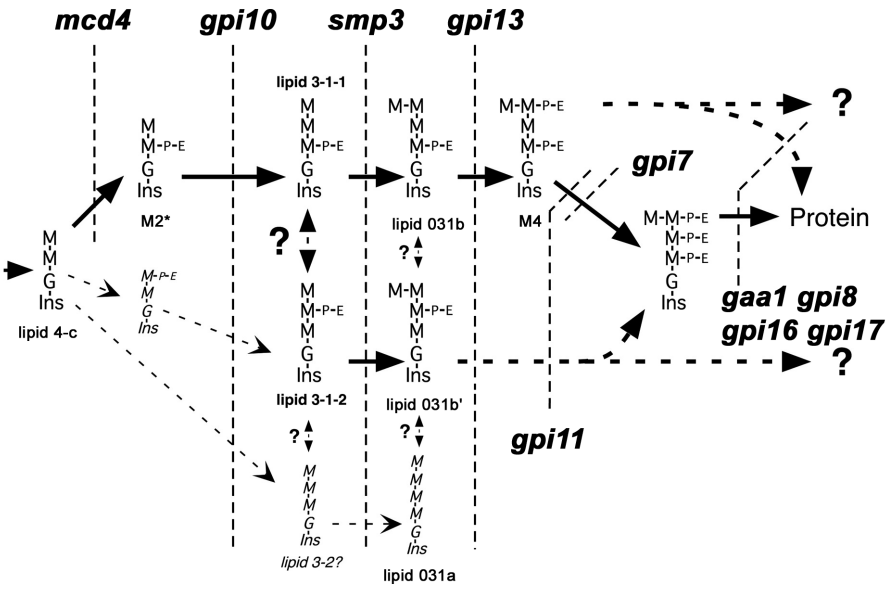

Figure 5-1. Model for a branched pathway for GPI biosynthesis in *Saccharomyces cerevisiae*.

Adapted from Grimme *et al.*, 2001. Dashed arrows indicate putative pathway branches, hypothetical intermediates are shown in italics. M, mannose; G, glucosamine; E, ethanolamine; P, phosphate; Ins, inositol.

The finding of Man_3- and Man_4-species having a single EthN-P either on Man1 or on Man2 in *smp3* mutant cells or Gpi13p-depleted cells respectively, support the idea of a pathway branching in yeast. As discussed by Grimme *et al.* (2001), it cannot be ruled out that the "EthN-P on Man2"-branch of the pathway is observed only in mutant cells, and therefore, under non-physiological conditions. The pattern of EthN-P modification observed in GPI-deficient mutants might have arisen from transesterification of the EthN-P side chains from one mannose residue to another. In this context, it is noteworthy that our preliminary characterization of the head group of lipid 031b from *Δgpi7 pGAL1,10-GPI13* cells (strain FBY1106) revealed the presence of a HF-sensitive substituent on Man2 to a similar extent as for lipid 031b from Gpi13p-depleted cells (result not shown). Assuming that Gpi7p really

is the transferase which adds a side chain on Man2, our result suggests that yet another enzyme than Gpi7p can attach a side chain onto Man2. However, we do not know if the side chain found on Man2 of lipid 031b is attached to the same position as the substituent of Man2 in CP2. We have shown that Gpi13p-depleted cells also accumulate a Man_4-species lacking EthN-P (lipid 031a). To date, we do not know if and how lipid 031a fits in the model for yeast GPI biosynthesis, and based on the data of Grimme *et al.* (2001), we believe that this lipid is a degradation product from lipid 031b. If lipid 031a is a degradation product, this could be an indication of aberrant processing of GPI intermediates in GPI-deficient mutants, an idea that would be consistent with the hypothesis by Grimme *et al.* (2001) that a branching of the GPI biosynthesis is only observed in mutants.

Future experiments should include *in vitro* tests with purified enzymes or complexes, in order to unambiguously determine the role of Gpi13p, Gpi7p, Mcd4p and Gpi11p in yeast GPI anchor biosynthesis.

6. Materials and Methods

Media and Materials

Growth media for *Saccharomyces cerevisiae* strains were:

YPD medium: 1% yeast extract (Difco), 2% casein hydrolysate (Life Technologies), 2% glucose (Fluka); YPGal medium: YPD with 2% galactose (Fluka) instead of glucose. SDC medium: minimal synthetic medium supplemented with 2% casein hydrolysate. Minimal synthetic medium without casein hydrolysate was supplemented with the required amino acids (20-400 mg/l), adenine sulfate and uracil. When indicated, yeast nitrogen base (Difco) was used to prepare minimal medium.

Reagents were purchased from the following sources: [2-^{3}H]*myo*-inositol, 20 Ci/mmol; [^{14}C]*myo*-inositol, 300 mCi/mmol; [2-^{3}H]mannose, 20 Ci/mmol; and uridine diphosphate-*N*-acetyl-D-glucosamine [glucosamine-6-^{3}H], 40 Ci/mmol were from Anawa Trading SA, Zürich; Kodak X-OMat films, anti-mouse and anti-rabbit IgG-peroxidase conjugates, monoclonal anti-GST antibodies, ATP, coenzyme A, tunicamycin, digitonin, CFW (fluorescent brightener 28) and GDP-mannose from Sigma; 6-aminocaproic acid and digitonin from Fluka; anti-c-*myc* antibodies from BAbCO, Richmond, CA); CNBr-activated sepharose 4B, glutathione sepharose 4B and ECL chemiluminescence kit and films from Amersham Pharmacia. 0.2 mm thick silica gel 60 plates from Merck; glass beads (0.45-0.55 mm) from Braun Melsungen; zymolyase 20-T from Seikagaku Corp.; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) from Biofinex; AG-501-X8 ion exchange resin from Bio-Rad laboratories; JBAM from Glyko; PI-PLC from ICN. PCR primers were from Microsynth GmbH, Balgach. Expand High Fidelity Taq DNA polymerase from Roche; restriction enzymes from New England Biolabs and Gibco. Anti-Wbp1p and anti-Och1p antibodies were a gift from Dr. Markus Aebi (ETH Zürich) and Dr. Y. Jigami (Ibarasaki, Japan), respectively. Yeast strains 17A-H42 and 521-17A-H42 were from A. Packeiser.

List of Saccharomyces cerevisiae strains

name	genotype	plasmid	source
FBY182	*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::kanMX4*		M. Benghezal
FBY413	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52*		A. Benachour
FY1679	*MATa/α ura3-52/ura3-52 leu2Δ1/+ trp1Δ63/+ his3Δ200/+*		Eurofan
FBY1102	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c*		I. Flury
FBY1103	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c YNR019w::kanMX4*		I. Flury
FBY1104	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 ykl165w::HIS3 pGAL1,10-YKL165w YNR019w::kanMX4*		I. Flury
FBY1105	*MATa/α ura3-52/ura3-52 leu2Δ1/+ trp1Δ63/+ his3Δ200/+ yll031c::kanMX4/YLL031c*		I. Flury
FBY1106	*MATa leu2 his3 ura3 gpi7::kanMX4 yll031c::HIS3 pGAL1,10-YLL031c*		I. Flury
FBY1107	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::YLL031c-13myc kanMX6*		I. Flury
FBY1108	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::YLL031c-3HA kanMX6*		I. Flury
FBY1120	*leu2 his sec18*		I. Flury
FBY1121	*leu2 his*		I. Flury
FBY1122	*trp1-Δ63 leu2 ura3-52 yll031c::HIS3 pGAL-YLL031c*		I. Flury
FBY1123	*sec18 ura3-52 trp1-Δ63, leu2 yll031c::HIS3 pGAL-YLL031c*		I. Flury
FBY1130	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52*	pUPRE	I. Flury
FBY1131	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c*	pUPRE	
FBY1132	*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::kanMX4*	pUPRE	
FBY1133	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 gpi7::GPI7-GST kanMX6*		I. Flury
FBY1134	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 ykl165w::HIS3 pGAL1,10-YKL165w YNR 019w::kanMX4*	pUPRE	I. Flury

name	genotype	plasmid	source
FBY1135	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52*	pBF639	I. Flury
FBY1136	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52*	pBF41	I. Flury
FBY1137	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52*	pBF115	I. Flury
FBY1138	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52*	pRS426	I. Flury
FBY1139	*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::kanMX4*	pBF639	I. Flury
FBY1140	*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::kanMX4*	pBF41	I. Flury
FBY1141	*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::kanMX4*	pBF115	I. Flury
FBY1142	*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7::kanMX4*	pRS426	I. Flury
FBY1143	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c*	pBF639	I. Flury
FBY1144	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c*	pBF41	I. Flury
FBY1145	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c*	pBF115	I. Flury
FBY1146	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 yll031c::HIS3 pGAL1,10-YLL031c*	pRS426	I. Flury
FBY1147	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 ykl165w::HIS3 pGAL1,10-YKL165w YNR 019w::kanMX4*	pBF639	I. Flury
FBY1148	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 ykl165w::HIS3 pGAL1,10-YKL165w YNR 019w::kanMX4*	pBF41	I. Flury
FBY1149	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 ykl165w::HIS3 pGAL1,10-YKL165w YNR 019w::kanMX4*	pBF115	I. Flury
FBY1150	*MATa leu2-Δ1 trp1-Δ63 his3-Δ200 ura3-52 ykl165w::HIS3 pGAL1,10-YKL165w YNR 019w::kanMX4*	pRS426	I. Flury
17A-H42	*MATa trp1-289 ura 3-52 leu2*		(Packeiser *et al.*, 1999)
521-17A-H42	*MATa trp1-289 ura 3-52 leu2 ssu21 (=mcd4)*		(Packeiser *et al.*, 1999)

List of plasmids

name	insert	vector type	selectable marker E.coli	Yeast	source
pBF115	*YLL031c (= GPI13)*	2µ	*Amp*	*URA3*	I. Flury
pBF41	*GPI7*	2µ	*Amp*	*URA3*	A. Benachour
pBF639	*MCD4*	2µ	*Amp*	*URA3*	I. Imhof
pET15-b			*Amp*		Novagen
pET-15b-YLL031c	aa 122-320 of Gpi13p		*Amp*		I. Flury
pRS426		2µ	*Amp*	*URA3*	(Christianson *et al.*, 1992)
pUPRE	*pUPRE-lacZ*	2µ	*Amp*	*URA3*	(Menzel *et al.*, 1997)
pYCGYLL031	*YLL031c (= GPI13)*	CEN	*Amp*	*URA3*	Euroscarf

List of oligonucleotides

name	sequence (5' → 3')	purpose
031A4	catcaatgaaagtcggtaagg	control insertion of GAL promoter
031S1-1842	gatgaaaaataatatacaaatcgcgaataaagaa atttcaacgtacgctgcaggtcgac	SFH-PCR for disruption of YLL031c, together with oligo S2031
165A4	agcaatcatagcaacatgacc	control insertion of GAL promoter
A2-031	gcgcgatgacgagcaaggt	control oligo for YLL031c disruption, tagging etc.
A3Gal	gagcagttaagcgtattactg	control insertion of GAL promoter
A4-031	gctgcagaaaagagatgc	control oligo for YLL031c disruption, tagging etc.
Abint-1	cccgcatgccatatggcaagcgataaggatgca	amplification of hydrophilic portion of YLL031c for cloning into pET15b for antibody raising
Abint-2	ggggtacccggatcccatgtccggcttcttaga	amplification of hydrophilic portion of YLL031c for cloning into pET15b for antibody raising
C1-GPI7	cttgtttgatgacggcatg	control oligo for insertion of GST tag
C1-myc	ctgacactgtggtcacagcc	control insertion of genomic myc tag
F2-GPI7	tggctggttacctgaattggccctcctttgcgctcttgat cggatccccgggttaattaa	tagging of GPI7 in the genome using plasmids described in (Longtine *et al.*, 1998)
F2-YLL031c	agtgggagattgattaagcacataaatgacatttttg gaaacggatccccgggttaattaa	tagging of YLL031c in the genome using plasmids described in (Longtine *et al.*, 1998)
K1031	catggtacaattgcaaagt	control disruption YLL031c
K2	gtcgcacctgattgcccg	control for presence of kanMX4 or kanMX6
L2-031	ttgaaatttctttattcgcgatttgtatattat	oligo L2 for LFH-PCR for disruption of YLL031c, antiparallel to genomic sequence in oligo 031S1-1842
L3-031	aatgacgaacttcatttccactctttacttacaaatata c	oligo L3 for LFH-PCR for disruption of YLL031c, antiparallel to the genomic sequence in oligo S2031

name	sequence (5' → 3')	purpose
R1-GPI7	catatatgtatacatatttagaccacgtgaacaattct atggaattcgagctcgtttaaac	tagging of GPI7 in the genome using plasmids described in (Longtine *et al.*, 1998)
R1-YLL031c	atatatagtatatttgtaagtaaagagtggaaatgaa gttcggaattcgagctcgtttaaac	tagging of YLL031c in the genome using plasmids described in (Longtine *et al.*, 1998)
S2031	tatagtatatttgtaagtaaagagtggaaatgaagttc gtcattatcgatgaattcgagctcg	SFH-PCR for disruption of YLL031c, together with oligo 031S1-1842

Experimental procedures

Protein extraction methods

Proteins for analysis by SDS-PAGE were extracted by the following methods:

- Rapid protein extraction method (Horvath and Riezman, 1994).

  Briefly, pelleted cells were denatured in reducing sample buffer (SB) (Laemmli, 1970) containing 20 mM EDTA by incubation at 95 °C for 5 min. Usually, a concentration of 1 OD_{600}/20 μl SB was used. A variation of this protocol were tested: addition of protease inhibitors (1 mM PMSF, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin) to SB and subsequent denaturation at 37 °C instead of 95 °C, but with this method, less proteins were obtained.

- Improved rapid protein extraction method (Kushnirov, 2000)

  Briefly, pelleted cells were incubated for 5 min at RT with 80 μl/OD_{600} 0.1 M NaOH. The tube was spun and SB containing 20 mM EDTA was added and proteins were denatured as described for the rapid extraction method.

- Denaturation in High Urea buffer

  Briefly, pelleted cells were denatured by incubating at 65 °C for 10-15 min in HU buffer (8M urea, 5% SDS, 200 mM Tris-HCl pH 6.8, 20 mM EDTA, bromophenol blue, 15 mg/ml DTT), usually at a concentration of 1 OD_{600}/20 μl HU. Important: add DTT immediately before use. This extraction method was also tested at denaturation temperatures of 0 °C, 37 °C and 95°C. At 65°C and 95 °C, the method yields more proteins than at lower temperatures, and seems also to yield more proteins than with reducing sample buffer.

Membrane association of Gpi13p

240 OD_{600} of exponentially grown cells were lysed in lysis buffer (20 mM HEPES pH 7.4, 0.1 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 30 µg/ml each Antipain, Pepstatin, Leupeptin) at 100 OD/ml by agitation with glass beads. Unbroken cells were removed by centrifugation at 600xg for 5 min. The supernatant was split in 6 aliquots of equal size. Aliquots were incubated for 40 min on ice in presence of 0.5 M NaCl, 0.8 M urea, 0.1 M Na_2CO_3, 1% Triton X-100, 1% SDS or control incubated with water. Samples were centrifuged at 100'000 x g for 1 h at 4 °C in AH-650 rotor. Pellet fractions were dissolved and denatured in High Urea buffer (8M urea, 5% SDS, 200 mM Tris-HCl pH 6.8, 20 mM EDTA, bromophenol blue, 15 mg/ml DTT) by sonication and incubation at 65 °C for 10 min. The supernatant was precipitated with trichloroacetic acid before denaturation in High Urea buffer.

Preparation of microsomal membranes and solubilization of membrane proteins and blue native polyacrylamide gel electrophoresis

Microsomal membranes were prepared as described (Fraering *et al.*, 2001), with the following modifications: FBY1133 and FBY413 cells were grown in YPDUA medium at 30°C. After thawing, 300 µl TM buffer (50 mM Tris-HCl pH 7.4, 0.2 M mannitol, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$ containing 1 mM DTT, and 1 mM PMSF, and 1 µg/ml of each chymostatin, pepstatin, antipain, and leupeptin) were added to every 100 µl of membrane suspension. DNA was digested with 0.2 mg/ml DNAse I (3000 U/mg; FLUKA, Buchs, Switzerland) for 45min at 25°C with shaking. Glycerol was adjusted to 10%, and the protein concentration was determined and diluted to 7 µg/µl with GTM-buffer (TM buffer with 10% glycerol). The solubilization of membrane proteins was achieved by adding digitonin (Sigma D5628, recrystallized prior to utilization) and 6-aminocaproic acid to final concentrations of 1.5% and 620 mM, respectively. After incubating for 45 min at 4°C with shaking, insoluble material was removed by centrifugation for 30 min at 40,000 rpm (100'000 x g) at 4°C in a TFT 90.4 fixed angle rotor (Kontron, Munich, Germany). The concentration of solubilized proteins was determined and samples were shock frozen in liquid nitrogen and stored at 80°C. Protein samples were then processed for blue native gel electrophoresis and were loaded onto 5-15 % polyacrylamide gradient gels as described (Fraering *et al.*, 2001). GST-tagged Gpi7p was detected with monoclonal anti-GST antibodies (Sigma, St. Louis, MO, USA), and western blots were revealed using the chemiluminescence ECL kit (Amersham Pharmacia).

Affinity chromatography of Gpi7p-GST

Analytical scale affinity purification of Gpi7p-GST was carried out essentially as described (Fraering *et al.*, 2001), except that microsomes from 1000 OD_{600} units of FBY1133 and FBY413 cells were solubilized in presence of 6-aminocaproic acid. 0.6 ml glutathione sepharose 4B (Amersham Pharmacia) was added to the lysate. TM buffer (0.2 M mannitol, 0.1 M NaCl, 50 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM PMSF, 1 mM DTT and 1 μg/ml of each chymostatin, pepstatin, antipain, and leupeptin) was added to the lysate in order to dilute digitonin to 0.5%. Beads were incubated overnight at 4 °C on a wheel and were sedimented by letting them stand for 1 h at 4 °C. The supernatant was carefully removed (unbound fraction). The glutathione sepharose 4B was washed three times with 5 ml TM buffer + 0.3% digitonin, rotating the tube on the wheel for 15 min, and letting stand for 1 h. The supernatants were carefully removed (washes 1-3). The bound proteins were eluted with 0.5 ml TM buffer + 0.3% digitonin + 10 mM reduced glutathione (Sigma) by gently rotating the tube for 30 min. The elution was repeated with 0.5 ml TM buffer + 0.3% digitonin + 20 mM reduced glutathione. pH of elution buffers was adjusted to pH 7.5 with Tris. Samples were processed for SDS-PAGE by precipitation with trichloroacetic acid before denaturation in 2x concentrated reducing sample buffer. Proteins were separated on a 6-15% gradient gel and stained with silver nitrate as described in Table 6-1.

Silver staining of proteins

The protocol described in Table 6-1 is adapted for staining of mini gels. Water of milli-Q quality has to be used.

Table 6-1. Silver staining protocol

step	solutions	incubation
Fix	40 ml methanol 10 ml acetic acid 50 ml water	10 min
Rinse	100 ml water	10 min
Fix and sensitize	40 ml ethanol 60 ml water 50 µl glutaraldehyde 50% 10 µl formaldehyde 37%	5 min
Rinse	40 ml ethanol 60 ml water	20 min
Rinse	100 ml water	20 min
Sensitize	20 mg sodium thiosulfate 100 ml water	1 min
Rinse	100 ml water per wash	2x 1 min
Silver stain	100 mg silver nitrate 100 ml water	20 min
Rinse	100 ml water	1 min, not more
Develop	2.5 g sodium carbonate 40 µl formaldehyde 37% 100 ml water	incubate gel with 25 ml until solution turns yellow (about 1 min), discard, develop with the rest of the solution (4-15 min)
Stop	5 ml acetic acid 95 ml water	5 min
Store	30 mg sodium carbonate 100 ml water	

This protocol is not indicated if tryptic digestion and mass spectrometry of proteins is foreseen.

Preparation of CFW-containing plates and growth test

Calcofluor white (CFW)-containing plates were made based on a protocol published by the EUROFAN network (http://mips.gsf.de/proj/eurofan/eurofan_2/n7/protocols.html). A stock solution of 100 mg/ml calcofluor white (Sigma F3543) was prepared by dissolving the dye in water with dropwise addition of KOH. A suspension of 0.67% yeast nitrogen base, 2% glucose, 3% agar, amino acids and adenine was sterilized, and buffered with 1% MES (2-[N-Morpholino]ethanesulfonic acid) pH 6.0 (from a 25% filter-sterilized stock solution). CFW

was added to a final concentration of 100 µg/ml before pouring the plates. Cells were taken from exponentially grown overnight cultures in galactose-containing medium lacking uracil. A tenfold dilution series of cell suspensions containing 20 OD_{600} units per ml was prepared. 2 µl of each dilution (10x, 100x, 1'000x, 10'000x and 100'000x) were spotted onto agar plates with or without CFW and growth of cells was scored after 3 d at 30 °C.

Preparation of X-Gal containing plates and growth test

Minimal medium (0.67% Yeast Nitrogen Base without amino acids; 2% glucose or galactose; all amino acids; adenine; 3% agar) was autoclaved. Sterile KH_2PO_4 pH 7 was added to 0.05 M after autoclaving. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was added to 40 µg/ml after autoclaving from a stock solution in DMF. FBY413, FBY1102 and FBY1104 cells were transformed with pUPRE, and grown to exponential phase in medium lacking uracil. Sequential 10x dilutions were spotted onto agar plates with or without X-Gal and growth of cells was scored after 4 d at 30 °C.

Subcellular fractionation and sucrose step

Exponentially grown cells from FBY413 (wild-type) and FBY1107 (*GPI13-myc*) were converted to spheroplasts, P13 and P100 pellet fractions were generated as previously described (Flury *et al.*, 2000), with the following modification: lysis buffer was 0.2 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM Hepes-KOH pH 6.8, 1 mM DTT, PMSF 20 µg/ml, antipain 5µg/ml, leupeptin 0.5 µg/ml, pepstatin 0.7 µg/ml. One half of the P13 fraction was placed on the top of a sucrose step (800 µl each of 1.5 M and 1.2 M sucrose in sucrose gradient buffer: 50 mM KOAc, 2 mM EDTA, 20 mM Hepes-KOH pH 6.8, 1 mM DTT, 1 mM PMSF). The gradient was spun at 85'000 g for 1 h in a AH-650 swing-out rotor. Five equivalent fractions were removed and precipitated by TCA before denaturation in HU buffer.

In vitro GPI biosynthesis in *mcd4* mutants

In vitro biosynthesis of GPIs with microsomes was performed as previously described (Flury *et al.*, 2000), with the following modifications: Strains 521-17A-H42 and 17-H42 were grown to exponential phase in YPD medium at 24 °C and shifted to 37 °C 2h prior to conversion to spheroplasts. For these strains, all subsequent incubations were at 37 °C.

The other strains were grown overnight in YPD or YPGal at 30 °C, and subsequent steps were performed at 30 °C. Microsomes were labeled as previously described (Flury *et al.*, 2000), except that labeling time with [^{3}H]UDP-GlcNAc was increased to 90 min. Lipids were extracted, desalted and analyzed by ascending TLC as previously described (Reggiori *et al.*, 1997).

Head group preparation

Lipids from strain 521-17A-H42 were purified by scraping 1x from silica gel plates. Soluble head groups were obtained by treatment of purified glycolipids with monomethylamine:water (10:3) for 1 h at 53 °C (Guillas *et al.*, 2001). Non-hydrolyzed lipids were removed by butanol extraction. The water-soluble headgroups were divided in two, and half of the sample was treated with JBAM as descibed. Both samples were then dephosphorylated by HF. Before paper chromatography in solvent mixture methylethylketone/pyridine/water (20:12:11), samples were *N*-acetylated and desalted over mixed-bed ion exchange resin AG-501-X8 as described (Puoti and Conzelmann, 1993). Radiolabeled chromatography standards were obtained as described (Benachour *et al.*, 1999).

Preparation of yeast genomic DNA

Yeast genomic DNA was prepared as described on the following website: www.fhcrc.org/labs/breeden/methods/genomic_DNAprep.html. Briefly, cells were grown overnight in 5 ml medium at 30 °C. Cells were spun and washed once with 1 ml water before resuspension in 500 µl lysis buffer (100 mM Tris-HCl pH 8, 50 mM EDTA, 1% SDS). 1.25 ml glass beads were added and cells were vortexed for 2 min. The liquid was recovered and transferred in a new tube. After addition of 275 µl 7 M ammonium acetate pH 7.0, the tube was first incubated at 65 °C for 5 min, and then on ice for 5 min. 500 µl chloroform was added, the tube was vortexed and spun for 2 min. The supernatant was removed and precipitated with 1 ml isopropanol. After a 5 min incubation at RT, the tube was spun for 5 min. The DNA pellet was washed with 70% ethanol, dried and dissolved in 50 µl water. Concentration and purity of DNA was determined by measuring the absorption at 260 nm and at 280 nm.

Purification of recombinant His-tagged Gpi13p and antibody production

The region corresponding to aa 122-320 of Gpi13p was PCR-amplified from yeast genomic DNA with the primers Abint-1 and Abint-2. The PCR fragment was digested with NdeI and BamHI and cloned into the MCS of the bacterial expression vector pET-15b (Novagen). The resulting plasmid pET-15b-YLL031c was used to transform the *E.coli* strain BL21 D3. Expression of the recombinant protein was induced in a 1 l culture with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 5 h at 37 °C. Bacteria were lysed by sonication in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 8 M urea, 1 mM PMSF). The lysate was centrifuged at 11'500 rpm in Sorvall centrifuge with SS34 rotor for 15 min at 4 °C. The lysate was incubated overnight at 4 °C with TALON resin (Clontech). The recombinant His_6-tagged protein was purified under denaturing conditions essentially as described in the manufacturers instructions (Clontech). From this step on, urea was omitted in the lysis buffer. Briefly, resin was washed twice with 10 ml lysis buffer and transferred to a 10 ml syringe. The resin was further washed 5 times with 1ml of lysis buffer containing 1 mM 2-mercaptoethanol. Then the resin was washed twice with 1 ml lysis buffer containing 10 mM imidazole. Bound proteins were then eluted with lysis buffer containing 50 mM (4 ml), 100 mM (4 ml), 150 mM (3 ml) and 200 mM (6 ml) imidazole. Protein concentration was determined with the Bradford method. A polyclonal antiserum was raised against this Gpi13p fragment by repeated intramuscular injections of 100–150 μg of recombinant protein into rabbits. 4 mg of the recombinant protein was coupled to CNBr-sepharose 4B (Amersham Pharmacia) according to the manufacturer's instructions, and antiserum was affinity-purified as described (Harlow and Lane, 1988).

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Declaration For The Faculty

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Sujet: Thèse présentée à l'Université de Fribourg, Suisse, pour l'obtention du grade de *Doctor rerum naturalium*

Mesdames, Messieurs,

Par la présente, je certifie que j'ai rédigé ma thèse "Glycosylphosphatidylinositol Membrane Anchors in *Saccharomyces cerevisiae:* Characterization of Proteins Involved in Side Chain Modifications" moi-même et sur la base d'un travail personnel sans aide illicite.

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List of publications

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

Flury I, Benachour A, Conzelmann A: **YLL031c belongs to a novel family of membrane proteins involved in the transfer of ethanolaminephosphate onto the core structure of glycosylphosphatidylinositol anchors in yeast**. *J Biol Chem* 2000, **275**:24458-24465.

Oral presentation

YLL031c, *GPI7* and *MCD4* represent a novel family of membrane proteins involved in the transfer of ethanolaminephosphate onto the core structure of GPI anchors in yeast. Swiss Yeast Meeting, Lausanne, November 5th, 1999

Poster presentation

Gpi13p is involved in ethanolaminephosphate transfer onto the core structure of GPI anchors in yeast. EMBO-WORKSHOP 5th Annaberg Conference on Protein Sorting and Processing in the Secretory Pathway, Goldegg, Austria, January 9-14, 2001; and Swiss-Japanese Scientific Seminar, Les Diablerets, March 14-17, 2001

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