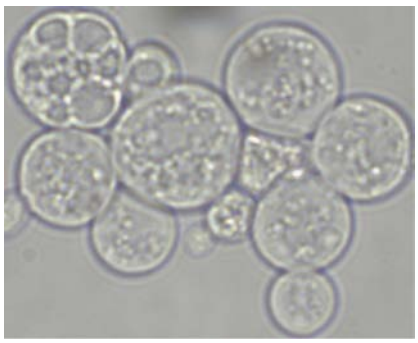
UNIVERSITE DE FRIBOURG
FACULTE DES SCIENCES
INSTITUT DE BIOCHIMIE

Functional analysis of the yeast *Saccharomyces cerevisiae* Gpi8 protein and characterization of the purified GPI-transamidase complex



Thèse

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Cover picture: *Saccharomyces cerevisiae* haploid cells depleted in Gpi16p, a new component of the GPI transamidase complex. The observed phenotype defines a structural rearrangement in the cellular organisation . See related article, p. 72

A mes grand-parents,
Maria,
Alfred,
Victorine et Robert

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ABBREVIATIONS

Asn	asparagine
COP	coatomer protein
CP	complete precursor
cpm	counts per minute
DAG	diacylglycerol
Dol	dolichol
Dol-P	dolicholphosphate
ER	endoplasmic reticulum
EtN	ethanolamine
EtNP	ethanolaminephosphate
FR-GPI	GPI-anchored folate receptor
Gal	galactose
GlcN	glucosamine
GlcNAc	N-acetyl-glucosamine
GlcN-PI	glucosaminyl-phosphatidylinositol
GlcNAc-PI	N-acetyl-glucosaminyl- phosphatidylinositol
GPI	glycosyl phosphatidylinositol
GPI-MT	GPI-Mannosyltransferase
GST	Glutathione S-transferase
Ins	*myo*-inositol
IPC	inositolphosphoceramide
IPTG	isopropylthiogalactoside
kDa	kiloDaltons
Ld	liquid-disordered
Lo	liquid-ordered
LPG	lipophosphoglycan
MAM	mitochondria-associated membranes
Man	mannose
Min	minute
MIPC	mannosyl inositolphosphoceramide
$M(IP)_2C$	mannosyl diinositolphosphoceramide
MSD	multi spanning domain
MS	mass spectrometry
OD	optical density
P	phosphate
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PI	phosphatidylinositol
pI	isoelectric point

PI-PLC	PI specific phospholipase C
PLAP	placental alkaline phosphatase
PNH	paroxysmal nocturnal hemoglobinuria
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
REC	recycling endosomal compartment
RgpB	gingipain R
RM	crude microsomal membranes
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SRP	signal recognition particle
TGN	*trans*-Golgi network
TLC	thin layer chromatography
TRIS	tris-(hydroxymethyl)-aminomethane
TMD	transmembrane domain
ts	temperature sensitive
UPR	unfolded protein response
VSG	variant surface glycoprotein
wt	wild type

ABSTRACT

Many glycoproteins of lower and higher eucaryotes are attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI). GPI anchoring of proteins is essential for the growth of *Saccharomyces cerevisiae* . *S.cerevisiae* contains about 70 open reading frames predicting GPI proteins and many of these have been found to be cell wall glycoproteins. The precursors of GPI anchored proteins have a classical signal sequence for import into the endoplasmic reticulum at their N-terminus and a GPI anchoring signal at their C-terminus which is necessary and sufficient to direct GPI addition. The C-terminal GPI anchoring signal (composed of a C-terminal hydrophobic domain separated by a short hydrophilic spacer from the cleavage/attachment ω site) is recognized and removed by a GPI transamidase, which replaces it by a preformed GPI. The GPI transferase is believed to act as a transamidase, i.e. to jointly remove the GPI anchoring signal and transfer the preformed GPI. Transamidase-deficient cells are expected to accumulate complete GPI's as well as GPI precursor proteins retaining the GPI anchoring signal. This phenotype is exhibited by two yeast mutants, *gaa1* and *gpi8*. Gpi8p and Gaa1p are essential components of the GPI transamidase which adds GPIs to newly synthesized proteins. *GPI8* is essential and encodes a 50 kDa type I ER membrane protein with a single membrane spanning domain (MSD), which is entirely lacking in *C. elegans* and some protozoan Gpi8p. Gpi8p has 25-28% homology to a family of cysteine proteinase (C13 family), one of which is able to act as a transamidase. The Cys and His residues, predicted to be active sites by sequence comparison with caspases indeed are essential and their mutation to Ala yields non-functional *GPI8* alleles. Yeast Gpi8p is fully functional without its C-terminal membrane spanning domain. This domain however helps to retain Gpi8p in the ER. Purification of the water soluble part of Gpi8p to homogeneity yields pure Gpi8p that has no detectable transamidase activity towards suitable peptides *in vitro*. *GAA1* is essential and encodes a 68 kDa ER protein with a large, hydrophilic, lumenal domain, followed by several MSDs and a cytosolic ER retrieval signal on its extreme C-terminus. After solubilization in 1.5 % digitonin and separation by blue native polyacrylamide gel electrophoresis, Gpi8p is found in 430 to 650 kDa protein complexes. These complexes

can be affinity purified and are shown to consist of Gaa1p, Gpi8p and Gpi16p (YHR188c). Gpi16p is an essential N-glycosylated transmembrane glycoprotein. Its bulk resides on the lumenal side of the ER, it has a single C-terminal transmembrane domain and a small C-terminal, cytosolic extension with an ER retrieval motif. Depletion of Gpi16p results in the accumulation of the complete GPI lipid CP2 and of unprocessed GPI precursor proteins. Gpi8p and Gpi16p are unstable if either of them is removed by depletion. Similarly, when Gpi8p is overexpressed, it largely remains outside the 430 to 650 kDa transamidase complex and is unstable. Overexpression of Gpi8p cannot compensate the lack of Gpi16p. Homologues of Gpi16p are found in all eucaryotes. The transamidase complex is not associated with the Sec61p complex and oligosaccharyltransferase complex required for ER insertion and N-glycosylation of GPI proteins, respectively. When GPI precursor proteins or GPI lipids are depleted, the transamidase complex remains intact. Recently, the identification of Gpi17p, another new component of the GPI transamidase complex was reported (Ohishi *et al*., 2001). Gpi17p is an essential N-glycosylated transmembrane glycoprotein. Its bulk resides on the lumenal side of the ER, and two transmembrane domains are predicted near the N- and C-termini. Depletion of Gpi17p results in the accumulation of the complete GPI lipid CP2.

The interaction of the hydrophilic, lumenal parts of each component of the transamidase complex, Gpi8p, Gaa1p, Gpi16p, and Gpi17p, with each other, was studied in a *GAL4*-based two hybrid system. From thus, a model of the macromolecular organization of the transamidase complex is proposed.

RESUME

Dans le monde des Eucaryotes inférieurs ou supérieurs, de nombreuses glycoprotéines sont ancrées dans leur membrane plasmique par l'intermediaire d'une ancre GPI (glycosylphosphatidylinositol). Cette modification post-traductionnelle est nécessaire pour la croissance et le développement de la levure *Saccharomyces cerevisiae*. Chez cet organisme, environ 70 cadres ouverts de lecture prédisent des proteins ancrées GPI, la majorité étant des glycoprotéines de la paroi cellulaire. Les précurseurs des protéines ancrées GPI possèdent du côté N-terminal un signal d'importation dans le reticulum endoplasmique (RE), et du côté C-terminal un signal d'ancrage GPI qui est nécessaire et suffisant pour l'addition d'une ancre GPI. Le signal d'ancrage GPI (composé d'un domaine C-terminal hydrophobique séparé du site de clivage ωpar une petite region hydrophilique) est reconnu et enlevé par une transamidase spécifique qui remplace ce signal par une ancre GPI préformée. Cette transférase à ancres GPI fonctionne telle une transamidase, à savoir qu'elle retire le signal d'ancrage et le remplace conjointement par une ancre GPI préformée. Les cellules déficientes pour cette transamidase accumulent des ancres GPI complètes (appelées lipides CP2), ainsi que des précurseurs de protéines conservant leur signal d'ancrage GPI. Ce phénotype est observé chez deux mutants de la levure, *gaa1* et *gpi8*. Gpi8p et Gaa1p sont deux composantes essentielles de la transamidase responsable du transfert des ancres GPI sur les protéines nouvellement synthétisées. *GPI8* est essentiel et code pour une proteine membranaire de type I de 50 kDa, caractérisée par un domaine transmembranaire absent chez *C. elegans* et chez certains protozoaires. Gpi8p partage 25-28% d'homologie de sequence avec une famille de protéases à cystéines (famille C13) qui présente une activité enzymatique propre aux transamidases. Les résidus Cys199 et His156 du site actif, identifiés après comparaison avec le site actif des caspases, sont essentiels; leur mutation en Ala donne lieu à des allèles *GPI8* non fonctionnels. La proteine Gpi8p de la levure est totalement fonctionnelle sans son domaine C-terminal transmembranaire. Ce domaine est cependant important dans la retention de la protéine Gpi8 dans le RE. Une proteine Gpi8 pure, obtenue après purification de la partie soluble de Gpi8p, ne présente aucune activité de transamidase, testée *in vitro* sur

des peptides substrats. *GAA1* est essentiel et code pour une protéine du RE de 68 kDa caractérisée par un large domaine luminal hydrophilique, et par plusieurs domaines transmembranaires prolongés en C-terminal par un signal cytosolique de retour vers le RE. La solubilisation dans 1.5 % de digitonine, suivie d'une séparation par électrophorèse sur gel natif de polyacrylamide, montre que Gpi8p est localisée dans des complexes protéiques de 430 à 650 kDa. Ces complexes ont été purifiés par des techniques de chromatographie d'affinité et les sous-unités analysées par des techniques de spectrophotometrie de masse. Ces analyses ont revelé la presence dans ces complexes de Gaa1p, Gpi8p, et d'une nouvelle protéine, Gpi16p (YHR188c). Gpi16p est une protéine transmembranaire, N-glycosylée, et essentielle. La protéine est située dans la partie luminale du RE, caractérisée par un domaine C-terminal transmembranaire prolongé par une petite partie cytosolique affichant un signal de retour vers le RE. La déplétion de Gpi16p résulte en l'accumulation dans le RE de lipides CP2, et de précurseurs protéiques non maturés. Gpi8p et Gpi16p sont instables si l'une ou l'autre des protéines est absente. Lors d'une surexpression de Gpi8p, la majorité des protéines se trouvant en dehors des complexes de 430 à 650 kDa est instable, puis rapidement dégradée. La surexpression de Gpi8p ne peut en aucun cas compenser le manque de Gpi16p. Des homologues de Gpi16p existent dans tous les organismes Eucaryotes. Le complexe de la transamidase ne se trouve associé, ni avec le complexe Sec61 requis pour le transfert des proteines dans le RE, ni avec le complexe de l' oligosaccharyltransferase requis dans la N-glycosylation. Lorsque les substrats de la transamidase, à savoir les précurseurs protéiques ainsi que les lipides CP2, sont dépletés, le complexe enzymatique reste stable. Récement, l'identification de Gpi17p, une autre nouvelle sous-unité du complexe de la transamidase à GPI a été rapportée (Ohishi *et al*., 2001). Gpi17p est une protéine transmembranaire, N-glycosylée, et essentielle. La protéine est située dans la partie luminale du RE, et présente deux domaines transmembranaires aux extremités N- et C-terminaux. La depletion de Gpi17p entraine une accumulation de lipides GPI complets CP2.

L'interaction entre les parties hydrophiliques et luminales de chacune des composantes du complexe de la transamidase (Gpi8p, Gaa1p, Gpi16p, et Gpi17p) a été étudiée par l'intermédiaire du système de double hybride basé sur le promoteur *GAL4*. Un modèle de l'organisation macromoleculaire du complexe de la transamidase est ici proposé.

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I. INTRODUCTION

It is just over a hundred years since Louis Pasteur demonstrated the essential participation of live yeast in fermentation processes (1879). The fact that strains of *Saccharomyces* (i.e.sugar-loving fungus) *cerevisiae* (i.e. from beer) are capable of reproducing both sexually and asexually in simple medium either in the presence or in the absence of oxygen and are non-toxic, has provided an ideal source of microbial biomass for research into cellular behaviour. Because of the general similarity between the mechanisms operating within the cells of so many living organisms, fundamental yeast research is of much wider application than fermentation research. Today, yeast *Saccharomyces cerevisiae* is commonly used as the model eucaryotic organism to study basic cellular phenomena. *S.cerevisiae* has a life cycle that is ideally suited to classical genetic analysis, and this has permitted construction of a detailed genetic map that defines the haploid set of 16 chromosomes. The complete genome of yeast S. *cerevisiae* was sequenced through a worldwide collaboration and released as first complete eucaryotic organism in 1996 (Goffeau *et al*., 1996). The sequence of 12068 kilobases defines 5885 potential protein-encoding genes, 10-20 % being predicted to be residents or passengers of the secretory pathway.

Yeast cells are delimitated from the outside world by a lipid bilayer containing proteins that vary markedly in the nature and extent of the interaction with the bilayer, and the cell wall, a glycan-rich envelope. The lipid bilayer of the plasma membrane constitutes a permeability barrier against the free exchange of water-soluble molecules. Metabolites are transported across this bilayer and signals that will control cell behavior are transmitted. The principal components of the plasma membrane are lipids, principally glycerophospholipids, sphingolipids, sterols, and proteins. Glycerophospholipids consists of two fatty acid acyl chains ester-linked to glycerol-3-phosphate with various substituents linked to the phosphoryl group, such as ethanolamine in phosphatidylethanolamine (PE), choline in phosphatidylcholine (PC), inositol in phosphatidylinositol (PI), and serine in phosphatidylserine (PS). Sphingolipids have a ceramide backbone which is composed of a long chain base phytosphingosine that is N-acylated with a hydroxy-C26 fatty acid.

S.cerevisiae contains three major sphingolipids: inositol phosphate ceramide (IPC), mannosyl-inositolphosphate ceramide (MIPC), and mannosyl-diinositolphosphate ceramide (M(IP)2C). Ergosterol is the major sterol detectable in plasma membrane extracts from yeast, in contrast to higher eucaryotes, in which cholesterol is the most abundant sterol. Specific functions were shown to be mediated by membrane proteins that cross the bilayer, including those carried out by solute-transport proteins (ex: the GAP general amino-acid permease), one or more ATPases to provide a proton-motive force across the membrane, enzymes that catalyse reactions in synthesis of wall components (ex: chitin synthase), and G-protein coupled receptor proteins such as α factor, or a factor receptors when yeast cells reside in water (for review, see van der REST *et al*., 1995).

The concentration of dissolved compounds inside the barrier is greater than that outside, so that an osmotic pressure difference exists across the barrier. To prevent water from passing in an uncontrolled fashion across the barrier, which would ultimatelly lead to cell lysis, S.*cerevisiae* has developed a tough cell wall. The wall makes up 15-30% of the dry weight of the vegetative cell, and its main components are mannoproteins (40%), β-1,3 and β-1,6 glucans (50-60%), while chitin makes up 1-2% of the wall. This cell wall does not only preserve the osmotic integrity of the cells, but also defines the morphology of the yeast cell during budding growth and during the developmental processes of mating and sporulation.

A growing yeast cell doubles its surface area every division cycle, stimulating the main function of the secretory pathway, which consists in generating and delivering new lipid and protein to the growing surface and the bud. Plasma membrane proteins are synthesized in the cytoplasm, transit the endoplasmic reticulum and the Golgi apparatus and travel to their final destination along the secretory pathway. The transport from one organelle to another is mediated by vesicular transport. Post-Golgi secretion vesicles are transported to a specific region of the plasma membrane, where membrane fusion takes place and the formation of the daughter cell starts during cell division, thus forming the bud (Novick *et al*., 1980). The attachment of surface proteins to the membrane occurs via transmembrane peptide sequences or via some type of lipid that is attached to the protein post-translationally. There are four types of lipid modifications for which signal sequences in the modified protein have been

identified: myristoylation, palmitoylation, prenylation (farnesylation or geranylgeranylation), and glycophosphatidylinositol (GPI) anchor addition. GPI anchor attachment is used to anchor proteins on the external surface of the plasma membrane whereas the other lipid modifications make cytosolic proteins adhere to the cytosolic side of membranes. TMD-anchoring of proteins is much more frequent except in protozoa. The discovery of GPI-linked proteins started with the isolation of bacterial phospholipases by Slein and Logan in the 1960s. In the late 1970s Ikezawa and Low independently showed that the enzyme alkaline phosphatase (AP) could be released from the plasma membrane of intact cells by treatment with bacterial phosphatidylinositol specific phospholipase C (PI-PLC). From this experience, they concluded that AP was bound to the membrane by some type of GPI anchor. In addition to his own studies, and by making highly purified PI-PLC available to many investigators, Low soon proved that AP was not unique and that many other proteins such as acetylcholinesterase (Low and Finean, 1977), Thy-1 (Low and Kincade, 1985), and trypanosomal variant surface glycoproteins (VSG) (Cross, 1984) are GPI anchored to the plasma membrane. Numerous glycoproteins of *S.cerevisiae* become attached to a GPI anchor and many appear in the cell wall or at the plasma membrane. The biosynthesis of GPI-anchored proteins follows the same basic rules in all eucaryotes, including yeast, and involves two independent metabolic pathways. One is the biosynthesis of the GPI moiety which consists of at least 10 reaction steps, and the other involves processing of the nascent protein and covalent attachment of the completed GPI moiety to yield the mature GPI protein.

I.1. Structure of GPI anchors

I.1.1. The core of GPI anchors

The structure of the core of the GPI anchor, **EtNP-6Manα1-2Manα1-6Manα1-4GlcNα1-6inositol-phospholipid** where EtNP, Man and GlcN are ethanolaminephosphate, mannose and glucosamine, respectively, is conserved in all eukaryotic cells (Ferguson *et al.*, 1999; Kinoshita *et al.*, 1997). This suggests that the GPI anchoring pathway has been established early in evolution and has been rigorously 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 (McConville and Ferguson, 1993).

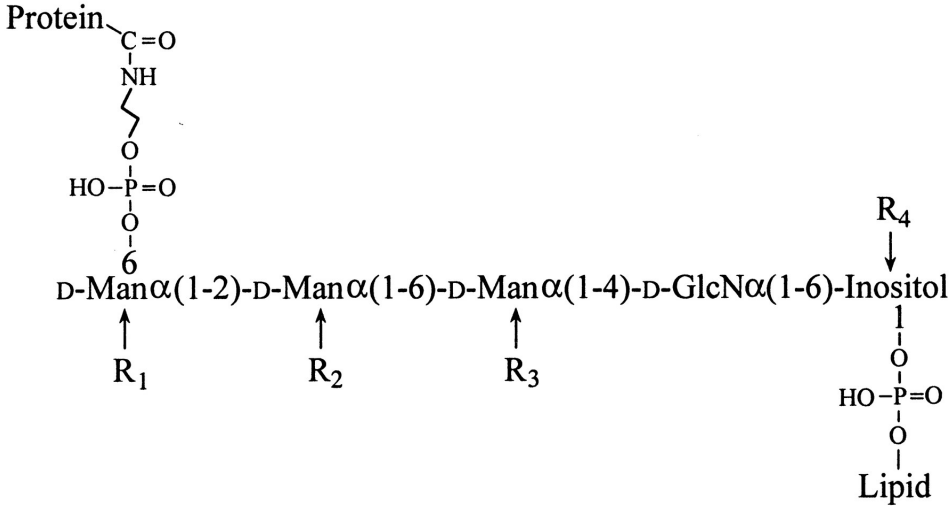


Fig.1. Structure of the conserved GPI anchor core from animals, protozoa, and yeast. The conserved core may carry additional substituents (R groups). Where present, R1 is usually α-Mannose, R2 is ethanolamine phosphate, R3 can be mono-, oligo-, poly-saccharides, or ethanolamine, and R4 is palmitate. The lipid can be a diacyl-, lysoacyl-, alkylacyl-, or lysoalkyl-glycerol or a ceramide.

I.1.2 Structure of the yeast *S.cerevisiae* GPI anchor

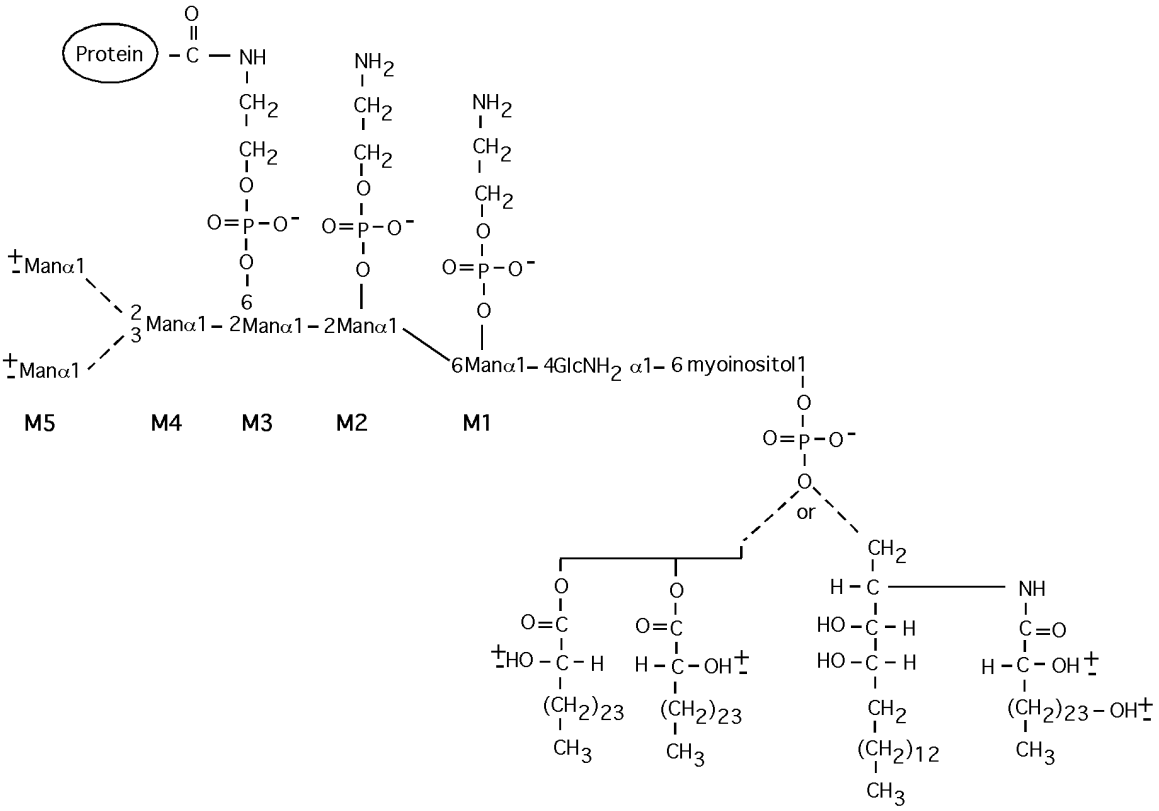


Fig.2. Structure of the *S.cerevisiae* GPI anchor.

The yeast carbohydrate core structure linking the protein to the lipid is identical to that of higher eukaryotes (Fig.1. Frankhauser *et al*., 1993). Figure 2 shows the characteristics of yeast GPI anchors which are:

(i) the presence of a side chain on the α1-2-linked mannose of the core oligosaccharide consisting of one or two mannose residues (M4 and M5 in Fig.below). 80% percent of the GPI anchors of wild type cells contain only M1 to M4. A fifth mannose (M5) is present in 20% of GPI anchors and is linked either α1-2 or α1-3 to M4 (Sipos *et al*., 1994).

(ii) the presence of additional phosphodiester linked residues on the first, second, and third mannose residue. The analysis of the GPI intermediate accumulating in *gpi10*, shows the presence of an ethanolamine phosphate on the α1-4 linked mannose (M1) (Canivenc-Gansel *et al*., 1998). Based on the analysis of M4, an abnormal GPI lipid accumulating in *gpi7* , Benachour *et al* (1999) proposed that Gpi7p adds an ethanolamine phosphate to the α1-6 linked mannose (M2). A third ethanolamine phosphate group, linked to the first α1-2 linked mannose (M3), forms the protein-ethanolamine bridge in GPI-anchored proteins (Taron *et al*., 2000).

(iii) the presence of two different types of lipid moieties, diacylglycerol or ceramide, both of which contain C26:0 fatty acids (Conzelmann *et al*., 1992). The ceramides consist of C18:0 phytosphingosine and a C26:0 fatty acid and are different from the main ceramides found in the abundant inositolphosphoceramides (IPCs) (Lester and Dickson, 1993). In both types of lipid moieties, the C26:0 may be hydroxylated on C2.

I.1.3 Structure of protein linked GPI-anchors from various organisms

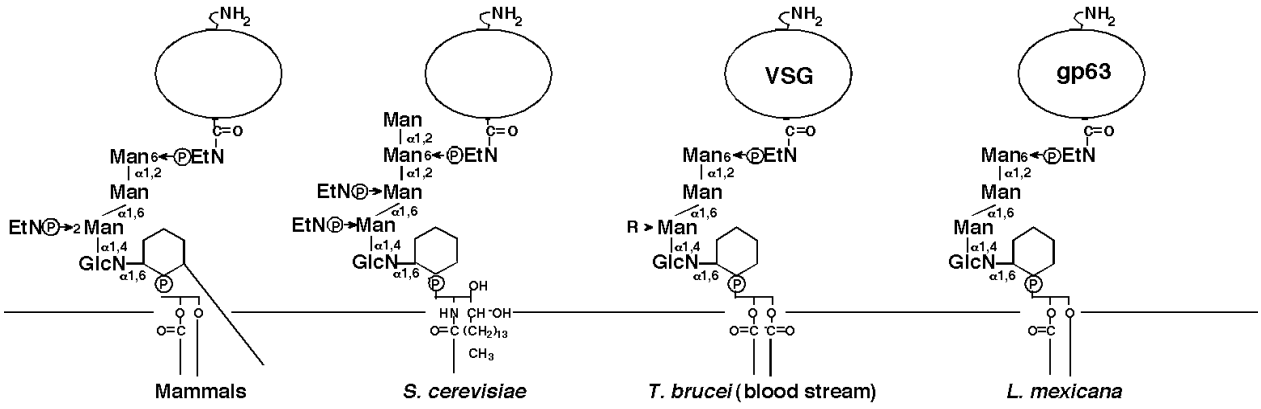


Fig.3. Structures of GPI-anchored proteins from various organisms. EtN, ethanolamine; GlcN, glucosamine; Man, mannose; P, phosphate; R, a side chaine consisting of several galactoses; VSG, variant surface glycoprotein. Derived from (Kinoshita and Inoue, 2000).

I.1.4 Structure of protein-free GPI-anchors from *Leishmania mexicana*

L.mexicana, which causes a cutaneous form of leishmaniasis, expresses three forms of GPI on the surface: those that act as membrane anchors of several surface proteins such as gp63 (see above), those that act as membrane anchors of abundant lipophosphoglycans, and those present as free GPI, termed GIPLs.

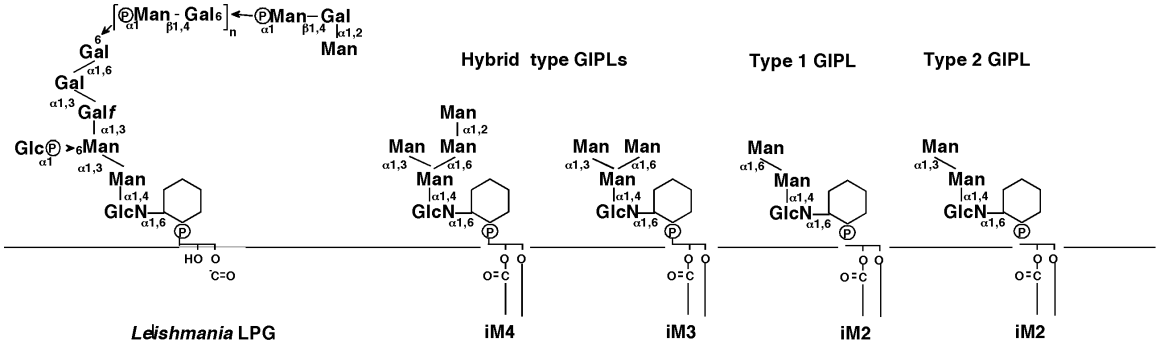


Fig.4. Structures of lipophosphoglycans and free GPIs from *L.mexicana*. Gal, galactose; Galf, galactofuranose; Glc, glucose; GlcN, glucosamine; Man, mannose; P, phosphate; LPG, lipophosphoglycan; GIPL, free GPI; iM2, iM3 and iM4, subtypes of GIPLs. Derived from (Kinoshita and Inoue, 2000).

I.2. GPI anchor biosynthesis

I.2.1 GPI anchor biosynthesis pathways in mammalian and *S.cerevisiae* cells.

Step 1: the generation of GlcNAc-PI from UDP-GlcNAc and phosphatidylinositol

The first step of GPI-anchor biosynthesis (step1 in Fig.5) is mediated by GPI-N-acetylglucosaminyltransferase (GPI-GnT). Mammalian GPI-GnT is a complex glycosyltransferase consisting of at least six proteins: PIG-A, PIG-C, PIG-H, GPI1, PIG-P and DPM2(Watanabe *et al*., 1998, 2000). In yeast, four genes, *GPI1*, *GPI2, YDR437w* and

GPI3/SPT14/CWH6 were shown to be required for this step. No homologs for the mammalian PIG-H and DPM2 were discovered so far in yeast. PIG-A is probably a catalytic component having homology to many glycosyltransferases (Bessler *et al*.,1994; Kawagoe *et al*.,1994). GPI1 is important but not essential for the activity of mammalian GPI-GnT. In yeast S.*pombe*, *Δgpi1* is not viable but growth could be restored by hGpi1p; *Δgpi1* of *S. cerevisiae* is viable but shows a temperature-sensitive (ts) phenotype for growth (Leidich and Orlean, 1996). This ts phenotype can be partially suppressed by the overexpression of Gpi2p indicating that the function of Gpi1p can be bypassed (Leidich *et al*., 1995). In mammalian cells, association of PIG-A with PIG-H is intact in the absence of GPI1, whereas association of PIG-C with the complex of PIG-A and PIG-H is inefficient. All these observations suggest that Gpi1p stabilizes the GPI-GnT complex (Hong *et al*., 1999; Watanabe *et al*., 1998). PIG-C, PIG-H and PIG-P are essential components but their functions have yet to be clarified.

DPM2 was previously found as a regulatory component of dolichol-phosphate-mannose (Dol-P-Man) synthase (Maeda *et al*., 1998). Isolated GPI-GnT contains DPM2 (Watanabe *et al*., 2000). DPM2 is not essential for GPI-GnT; however, its activity is enhanced threefold by the presence of DPM2, indicating that DPM2 positively regulates GPI-GnT.

Step 2: the generation of GlcN-PI from GlcNAc-PI

The second step is mediated by N-acetylglucosaminylphosphatidylinositol (GlcNAc-PI) de-N-acetylase (Figure 5). PIG-L and its yeast homologue *GPI12* discovered by homology searches were shown to be essential in the second step; purified PIG-L has the enzyme activity *in vitro* (Watanabe *et al*., 1999). No deacetylase activity could be detected in the GPI-GnT complex indicating that first and second steps are spacially separated (Nakamura *et al*., 1997). The Pig-lp is a type II membrane protein localized on the cytosolic face of the ER. The GlcNAc-PI analogue containing 2-*O*-octyl-D-*myo* –inositol and the GlcNAc-PI analogue containing L-myo-inositol are substrates for the trypanosomal enzyme but not for the HeLa enzyme, indicating that human enzymes have narrower substrate-specificity than that of trypanosomes (Sharma *et al*., 1999).

Step 3: the acylation of the inositol ring of GlcN-PI to generate GlcN-acyl-PI.

Step 3 is the acylation, mostly palmitoylation, of the *myo* -inositol of glucosaminylphosphatidylinositol (GlcN-PI) at position 2 by a non identified enzyme. In contrast to trypanosomes, this step is obligatory in both mammals and yeast (Herscovics and Orlean, 1993; Doerrler *et al*., 1996; Guther and Ferguson., 1995). It is not known whether acyl-CoA is the immediate acyl donor (Costello and Orlean, 1992) or whether the palmitoyl group is transferred by a CoA-dependent transacylation since both reactions were suggested to occur in mammals, whereas in yeast cells only a palmitoyl-CoA dependent transfer was described (Stevens and Zhang., 1994). Inositol acylation renders GPI-anchored proteins PI-PLC resistant. However, most of the eucaryotic GPI-anchored proteins are PI-PLC sensitive indicating that this acylation is transient (Roberts *et al*., 1988; Nakashima *et al*., 1992). Recently, two activities of palmitoyl transfer on GPI anchors were identified in rodent microsomes (Doerrler and Lehrman, 2000).

Step 4 and 5: translocation of the GlcN-acyl-PI from the cytoplasmic to the lumenal side and first mannosylation to generate Man-GlcN-acyl-PI.

The first mannose is transferred from Dol-P-Man to position 4 of GlcN (Figure 5) to generate Man-GlcN-acyl-PI. The recently cloned PIG-M gene (*S.cerevisiae* homolog *GPI14*) encodes the GPI α1-4 mannosyltransferase (GPI-MT)-I responsible for this step (Y.Maeda *et al*., 2001). PIG-M has a functionally essential DXD motif (a motif found in many glycosyltransferases) within a lumenal domain, suggesting that the transfer of the first mannose occurs on the lumenal side of the ER. It was shown that the biosynthesis of GlcN-PI occurs on the cytoplasmic side; therefore, GlcN-PI and/or GlcN-acyl-PI should translocate from the cytoplasmic side to the lumenal side. This would be mediated by a not yet identified "flippase".

Step 6: the modification of Man-GlcN-acyl-PI with a phosphoethanolamine side chain to generate EtNP-Man-GlcN-acyl-PI.

The first mannose is modified by ethanolaminephosphate (EtNP) in both mammalian and

S.cerevisiae systems (Sütterlin *et al*., 1998; Canivenc-Gansel *et al*., 1998). *S.cerevisiae MCD4* (Gaynor *et al*., 1999) and Mammalian PIG-N (isolated by homology searches with *MCD4* ; Hong *et al*., 1999) are involved in this reaction. Disruption of PIG-N in mouse F9 cells resulted in a lack of side- chain modification on the first mannose, whereas the surface expression of GPI-anchored proteins was only slightly decreased. Therefore, this side-chain modification is not essential for later reactions (Hong *et al*., 1999). In contrast, *MCD4* is essential for the growth of *S.cerevisiae*, indicating that this modification is critical for yeast (Gaynor *et al*., 1999). PIG-N and *MCD4* are probably the EtNP transferase genes. They have sequence motifs that are conserved in various nucleotide phosphodiesterases and pyrophosphatases within an ER lumenal domain. Furthermore, in yeast, *MCD4* has two homologs: *GPI7* (involved in side-chain modification of the second mannose (Benachour *et al*., 1999)) and *GPI13* (involved in transfer of EtNP to the third mannose (Taron *et al*., 2000; Flury *et al*., 2000)). *MCD4, GPI7, and GPI13* form a gene family and share the above motifs.

A terpenoid lactone, YW3548, produced by a fungus, inhibits the addition of EtNp to the first mannose (Hong *et al*., 1999). It kills *S.cerevisiae* at micromolar concentrations (Sütterlin *et al*., 1998; Hong *et al*., 1999), but has no effect on GPI biosynthesis in *T.brucei* because the first mannose in GPI of *T.brucei* is not modified.

Step 7 and 8: additions of the second and third mannoses.

The second mannose is transferred from Dol-P-Man to position 6 of the first mannose in EtNP-Man-GlcN-acyl-PI (Figure 5) which results in Man-(EtNP)Man-GlcN-acyl-PI (M2). The α 1-6 mannosyltransferase, which transfers the second mannose (GPI-MT-II), has not been identified.

The third mannose is transferred from Dol-P-Man to position 2 of the second mannose by an α1-2 mannosyltransferase (GPI-MT-III) forming Man-Man-(EtNP)Man-GlcN-acyl-PI. Mammalian PIG-B and *S.cerevisiae* Gpi10p (Sütterlin *et al*., 1998) are most likely GPI-MT-III itself. Corresponding mammalian and yeast mutants accumulate an M2 precursor. The structure of the intermediate accumulated by the mutant *gpi10-1* revealed the structure Man-(EtNP)Man-GlcN-acyl-PI (M2) (Canivenc-Gansel *et al*., 1998; Sütterlin *et al*., 1998). This

structural data combined with the data on ethanolaminephosphate transferases in yeast suggest that the ethanolamine phosphate on the second mannose is transferred most probably at a later stage occuring after the transfer of a third mannose (Flury *et al*., 2000).

Step 9: the addition of EtNP to the third mannose to generate EtNP-Man-Man-(EtNP)Man-GlcN-acyl-PI (termed H7), a GPI species transferred to proteins.

The EtNP that links GPI to proteins is transferred from phosphatidylethanolamine to position 6 of the third mannose. Two gene products, PIG-F and PIG-O, are involved in this step (Hong *et al*.,2000). PIG-O is a mammalian homologue of yeast Gpi13p (Taron *et al*., 2000) and is likely to be an essential catalytic component of the EtNP transferase. Gpi13p was identified as an essential protein that is homologous to Gpi7p and Mcd4p (Taron *et al*., 2000; Flury *et al*., 2000). Gpi13p-deficient cells accumulate an intermediate with the structure Man_4-GlcN-acyl-PI with or without EtN-P on the first mannose (Taron *et al*., 2000). PIG-F, and its yeast homologue Gpi11p, are very hydrophobic proteins that have no significant homology to other proteins of known functions. Gpi11p-deficient cells accumulate a biosynthetic intermediate which shows a lipid profile that resembles to that of cells lacking Gpi7p. Possible roles of Gpi11p in the supply of EtN-P were discussed (Taron *et al*., 2000). PIG-O and PIG-F form a complex and the expression level of PIG-O is dependent upon PIG-F (Hong *et al*.,2000).

Step 10: side-chain modification of the second mannose by EtNP to generate EtNP-Man-(EtNP)Man-(EtNP)Man-GlcN-acyl-PI (termed H8)

The second mannose of H7 can be modified by side-chain EtNP at position 6 to generate H8. Both H7 and H8 are potentially direct precursors of protein-bound anchors. They also exist as free GPI on the cell surface (Baumann *et al*., 2000). *S.cerevisiae* mutant *gpi7* accumulates a GPI intermediate that lacks a side-chain modification of the second mannose (Benachour *et al*., 1999). Gpi7p was isolated in a screen for yeast mutants unable to present The GPI anchored cell wall protein α−agglutinin on their surface (Benghezal *et al*., 1995; Benachour *et al*., 1999). In contrast to the related, putative EtN-P transferases Mcd4p and Gpi13p, Gpi7p is

not essential for GPI anchoring, but its deletion renders cells hypersensitive to Calcofluor White, indicating cell wall fragility. There is a human homologue of *GPI7* in the database, but whether it is involved in this modification is yet to be determined.

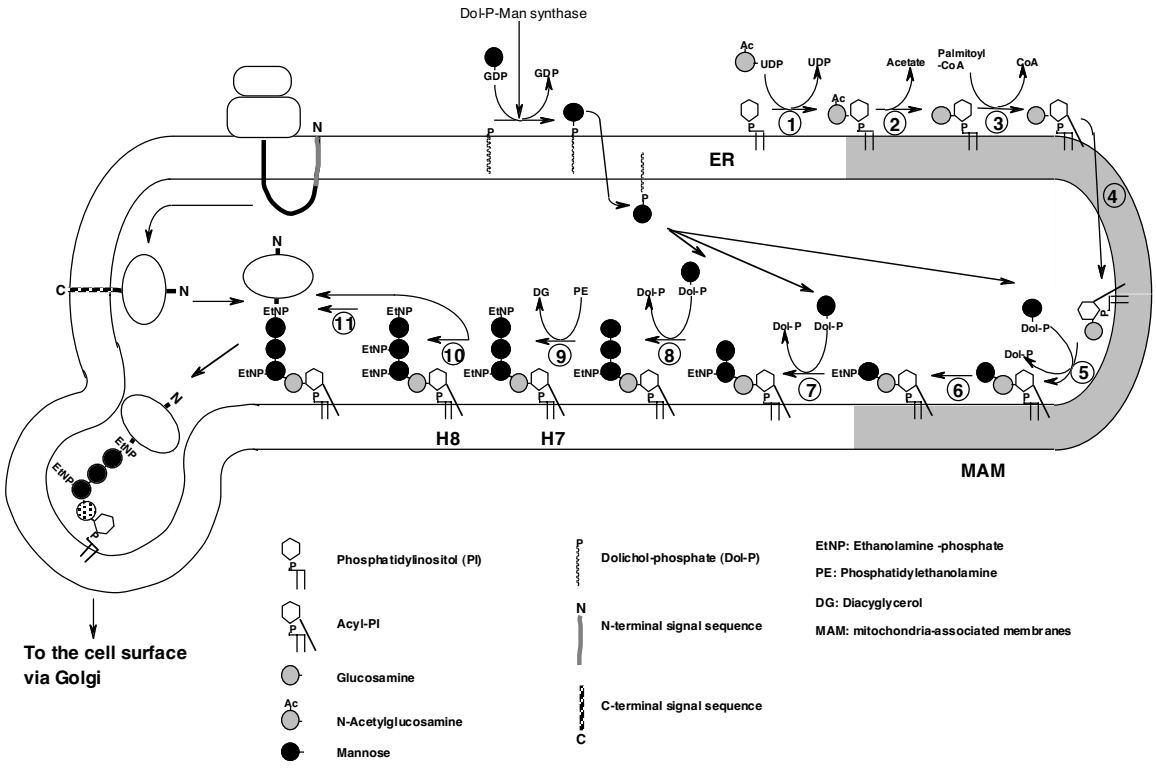


Fig.5 . GPI anchor biosynthesis and transfer to proteins in mammalian cells. Biosynthesis of GPI is initiated on the cytoplasmic side of the ER by the transfer of *N*-acetylglucosamine to phosphatidylinositol (step1). GlcN-PI and/or GlcN-acyl-PI precursors (steps 2-3) translocate from the cytoplasmic side to the lumenal side of the ER by a putative flippase (step 4). Steps 2 to 6 mainly occur in the mitochondria-associated membranes (MAMs). Pre-assembled GPI is transferred to proteins bearing a carboxy-terminal GPI-attachment signal sequence (step 11). GPI anchored proteins are incorporated into vesicles and transported to the cell surface. DG, diacylglycerol; Dol-P, dolichol-phosphate; PE, phosphatidylethanolamine. (Kinoshita and Inoue, 2000).

I.2.2. Genes involved in GPI anchor biosynthesis in yeast and mammals.

Table 1. Genes involved in GPI anchor biosynthesis in yeast and mammals.

Step	Enzyme	yeast gene	mammalian gene	references
1	**PI-GlcNAc Transferase**	*SPT14*	PIG-A	Leidich *et al*., 1995 Miyata *et al*., 1993
		GPI1	hGPI1	Leidich and Orlean,1996 Watanabe *et al*., 1998
		GPI2	PIG-C	Leidich *et al*., 1995 Inoue *et al*., 1996
		*	PIG-H	Kamitani *et al*., 1993
		YDR437w	PIG-P	Watanabe *et al*., 2000
		*	DPM2	Watanabe *et al*., 2000
2	**GlcNAc-PI-de-N-acetylase**	*GPI12*	PIG-L	Watanabe *et al*., 1999
3	**GlcNAc-PI Acyl-transferase**	?	?	
4	**Flippase**	?	?	
5	**GlcN-(acyl)PI α 1-4 Mannosyl-transferase M1 synthesis**	*GPI14*	PIG-M	Maeda *et al*., 2001 Kim *et al*., unpublished
6	**M1 EtN-P transferase**	*MCD4*	PIG-N	Gaynor *et al*., 1999 Hong *et al*., 1999
7	**M1 α 1-6 Mannosyl-transferase M2 synthesis**	?	?	

8	**M2 α 1-2 Mannosyl-transferase** **M3 synthesis**	*GPI10/ YGL142C*	PIG-B	Benghezal *et al*., 1995 Sütterling *et al*., 1998 Canivenc-Gansel *et al*., 1998 Takahashi *et al*., 1996
9	**Mannose 3 EtN-P transferase**	*GPI13/ YLL031c* *GPI11*	PIG-O PIG-F	Taron *et al*., 2000 Flury *et al*., 2000 Ohishi *et al*., 1996 Orlean *et al*., 2000
10	**Mannose 2 EtN-P transferase**	*GPI7*	h *GPI7*	Benachour *et al*.,1999 Kinoshita et al., *in preparation*
constitutive synthesis of dolichol phosphate mannose		*DPM1* * *	DPM1/ PIG-E DPM2 DPM3	Orlean *et al*., 1988 Tomita *et al*., 1998 Maeda *et al*., 1998 Maeda *et al*., 2000

Genes which have no homologues are marked by an asterisk (*). Unidentified genes are marked by a question mark (?).

I.2.3. The different characteristics of the GPI-biosynthesis pathways in *S.cerevisiae*, mammals, and *T.brucei*.

Unlike in mammalian and *T.brucei* cells, a fourth mannose is added to position 2 of the third mannose in *S.cerevisiae*. The *smp3* mutant is defective in the addition of the fourth mannose and accumulates GPI intermediates bearing three mannoses (Taron *et al*., 2000). These intermediates lack EtNP on the third mannose, indicating that the addition of the fourth mannose is prerequisite for the addition of EtNP to the third mannose. Moreover, unlike in yeast and mammalian cells, in the *T.brucei* system, transfer of the first mannose occurs before acylation of inositol (Smith *et al*., 1999), and the first mannose is not modified by an EtN-P side chain. *T.brucei* express fatty acid synthase that preferentially generates myristate, which is then used to remodel GPI by replacing both fatty acyl chains before the addition of the anchor to the proteins (Morita *et al*., 2000). In *S.cerevisiae*, GPI remodelling from diacylglycerol to ceramide has also been shown (Conzelmann *et al*., 1992; Reggiori *et al*.,

1997; Sipos *et al*., 1997). A remodelling mechanism has also been proposed to explain the prevalence of sn-1-alkyl, 2-acylglycerol in mammalian cells (Singh *et al*., 1994).

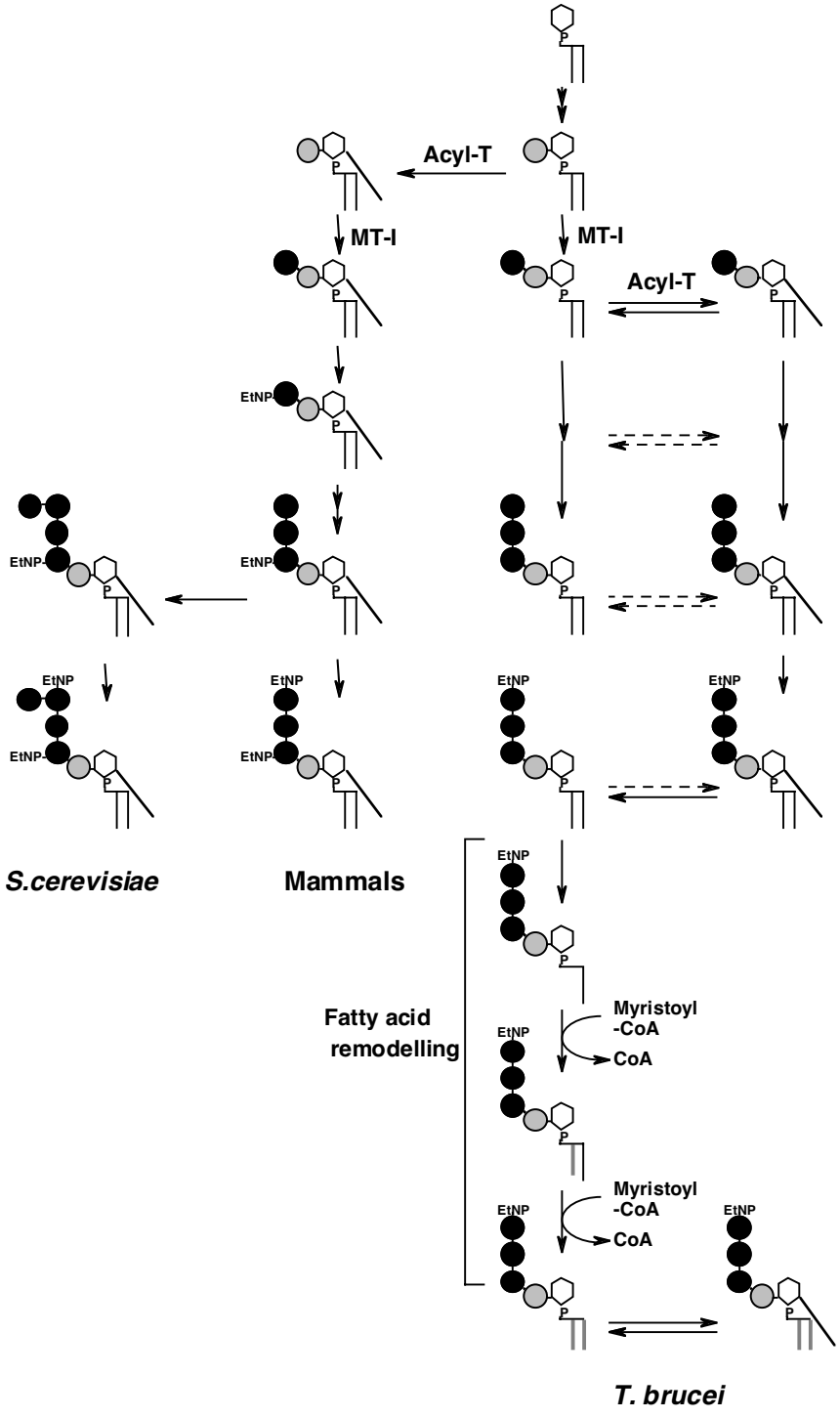


Fig.6. GPI biosynthesis pathways in *S.cerevisiae*, mammals, and *T.brucei*. (Kinoshita and Inoue, 2000).

From current knowledge, approximatively 6000 proteins are expressed by *S. cerevisiae*; by considering the amino-acid sequence, more than 1800 genes appear to encode proteins with one or more predicted transmembrane domains. Finally, 10 to 20% of all yeast proteins are either residents or passengers of the secretory pathway.
A growing yeast cell doubles its surface area every division cycle. The main function of the secretory pathway is to generate and deliver new membrane and protein to the growing surface and the bud, as well as to the vacuole. Secretory proteins, like GPI-anchored proteins, begin their synthesis in the cytosol and must be subsequently targeted to their functional compartment by specific signal sequences present in the newly synthesized polypeptide chain.

I.3. Protein Translocation Across the Membrane of the Endoplasmic Reticulum

I.3.1. Signal sequences

Proteins entering the secretory pathway contain an ER targeting signal, commonly referred to as the signal sequence. Eucaryotic signal sequences usually have a short amino-terminal region that often carries a net positive charge, a hydrophobic region that consists of a segment of 7-15 residues that does not contain charged residues and is enriched in hydrophobic residues, and a signal peptidase cleavage region of three to seven polar residues (Blobel and Sabatini, 1971; Milstein *et al*., 1972; von Heijne., 1985, 1990).

A direct experimental test of the limits of variation of signal sequences was performed by determining what kinds of sequences could functionally replace the signal sequence of invertase. About 20% of essentially random amino acids, forming hydrophobic random sequences, will direct secretion of invertase, showing that many different sequences have the capacity to target a protein to the translocation machinery (Kaiser *et al*., 1987).

A **cotranslational** mechanism involving a cytosolic ribonucleoprotein complex, Signal Recognition Particle (SRP), and its cognate receptor (SR) in the ER membrane has been described. Mutant yeast cells lacking SRP are viable albeit with a severe growth defect and defects in the translocation of some proteins (Brown et al., 1994; Hann and Walter, 1991, Stirling and Hewitt, 1992). This observation implies that targeting to the ER can occur via a second pathway in vivo. This second pathway represents a **post-translational** mechanism which has been demonstrated in vitro for precursors as large as 250 residues (Hansen and Walter, 1988; Singh *et al*., 1996).

I.3.2. The SRP dependent cotranslational translocation.

I.3.2.1. SRP and SRP Receptor

SRP was discovered as a cytosolic factor that is needed to restore translocation to mammalian microsomal membranes that have been extracted with high salt to remove peripheral proteins (Walter and Blobel, 1980). The yeast homolog of SRP is a complex that is composed of a 16S RNA and six polypeptides: Srp54p, which is a component of a 16S cytosolic ribonucleoprotein complex that contains a RNA moiety encoded by SCR1 (Felici *et al*., 1986; Hann and Walter, 1991), Sec65p (Hann *et al*., 1992; Stirling and Hewitt, 1992), and four additionnal proteins, Srp14p, Srp21p, Srp68p and Srp72p, isolated by immunoaffinity chromatography purification of the yeast SRP complex (Brown *et al*., 1994). Controlled inactivation of SRP54 or SEC65 shows severe translocation defects for the integral membrane proteins dipeptidyl-aminopeptidase B (DPAP-B) and Pho8p, partial translocation defects for Och1p, invertase, prepro- factor and Kar2p, and no defect for Gas1p, Pdi1p and CPY (Hann and Walter, 1991; Hann et al., 1992; Stirling and Hewitt, 1992; Ng *et al*., 1996). These findings indicate that there are two avenues for protein translocation into the ER, one dependent on SRP (Fig.7), and the other independent of SRP (Fig. 8).

The two subunits of the yeast SRP receptor have been identified by homology with their mammalian counterparts. *SRP101* encodes SRα , a 69-KD peripheral membrane protein

(Ogg *et al*., 1992) that is bound to the ER membrane by SRβ, a 30-KD integral membrane protein (Miller *et al*., 1995).

SRP recognizes signal sequences and selectively binds to ribosomes that are translating secretory proteins at the stage in chain elongation when the signal sequence emerges from the ribosome. The complex of nascent chain, ribosome, and SRP can then engage the membrane by binding to the SRP receptor. The passage of the polypeptide chain accross the membrane requires the Sec61p complex described below (Figure 7).

I.3.2.2. The Sec61p complex.

This complex is composed of Sec61p (Rothblatt *et al*., 1989), Sss1p which was isolated as a dosage-dependent suppressor of *sec61* mutations (Esnault *et al*., 1993), and Sbh1p, which was identified by its homology with the mammalian protein and was found to copurify with yeast Sec61p and Sssp (Panzner *et al*., 1995). A second multimeric protein complex closely related to the Sec61p complex has been identified in yeast. This complex is composed of Ssh1p (a homolog of Sec61p), Sbh2p (a homolog of Sbh1p) , and Sss1p (a component common to both complexes) (Finke *et al*., 1996).

The trimeric Sec61p complex in both yeast and mammals has been widely implicated as a component of the pore as a result of crosslinking of proteins at various stages of translocation (Görlich *et al*., 1992).

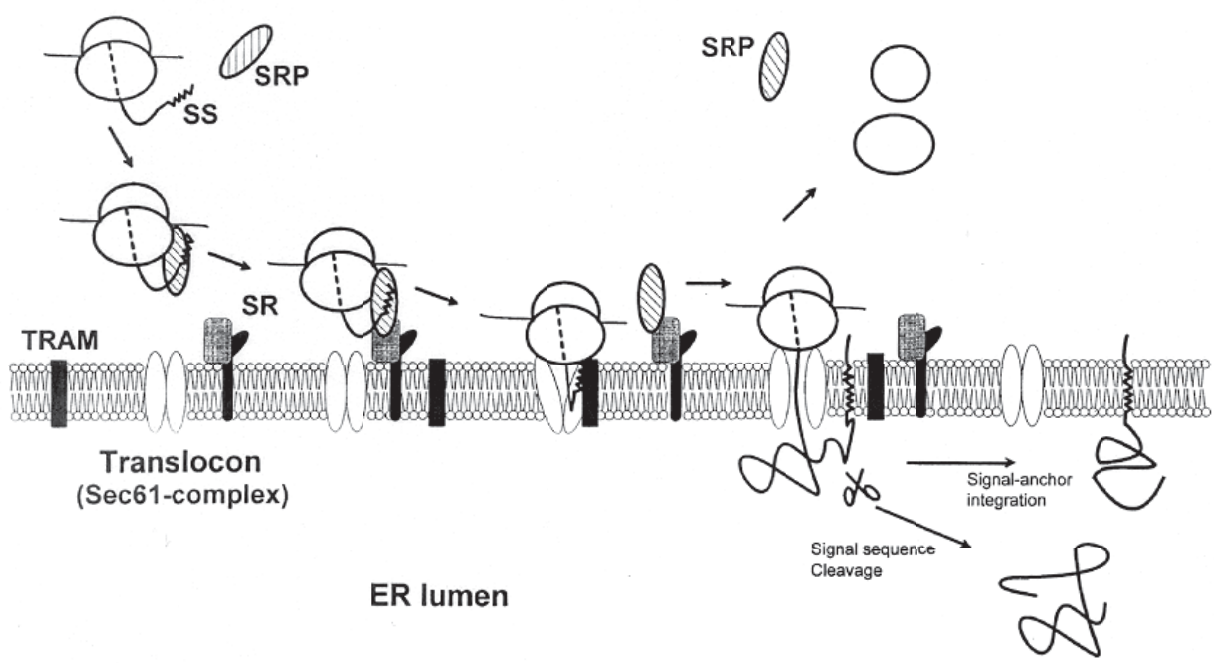


Fig.7. The mammalian SRP dependent cotranslational translocation. (Wilkinson *et al*., 1996).

I.3.3. Post-Translocation in Yeast

Genetic and biochemical studies in yeast have identified several additional ER components required for a post-translational translocation mechanism. These include the integral membrane proteins Sec62p, Sec63p, and Sec71p, the peripheral membrane protein, Sec72p, and the ER lumenal proteins Kar2p and Lhs1p (Craven *et al*., 1996; Deshaies *et al*., 1991; Feldheim and Shekman, 1994; Green *et al*., 1992; Kurihara and Silver, 1993; Sadler et al., 1989; Toyn *et al*., 1988; Vogel *et al*., 1990). Of these, Sec62p, Sec63p, Sec71p and Sec72p form a stable tetrameric complex which can also be found in association with the trimeric Sec61p complex (Brodsky and Shekman, 1993; Feldheim and Shekman, 1994; Deshaies *et al*., 1991).

The mechanism by which post-translationally translocated polypeptides are initially targeted to the ER remains uncertain. Evidence suggests that the translocation of some precursors may depend on the activities of the cytosolic chaperones Ssa1-4 and Ydj1p, which are believed to maintain precursors in a loosely-folded conformation required for their productive interaction

with the membrane translocon (Figure 8) (Becker *et al*., 1996; Caplan *et al*., 1992); Chirico *et al*., 1988; Cyr *et al*., 1994, 1992; Deshaies *et a*l., 1988). Genetic and biochemical data indicate that the "DnaJ box" of Sec63p interacts physically with Kar2p (Brodsky and Shekman, 1993).

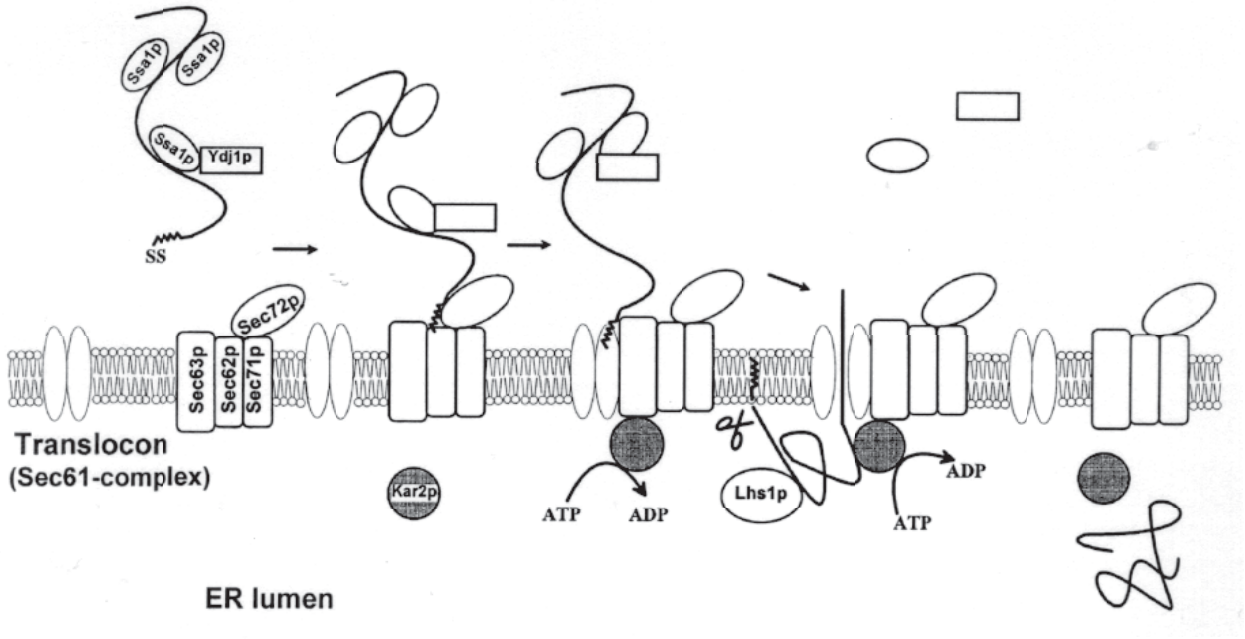


Fig.8. Mammalian Post-Translocation in the ER. (Wilkinson *et al*., 1996).

I.3.4. Kar2p

Kar2p was first identified in mutants that failed to fuse haploid nuclei during mating, a process known as karyogamy (Polaina and Conde, 1982)

In the post-translational translocation mechanism, precursors bind in an ATP-independent manner to a putative signal sequence receptor complex consisting of Sec62p, Sec71p, Sec72p and possibly Sec63p; Kar2p and Sec63p then mediate, in an ATP-dependent reaction, the transfer of the precursors from this complex to the pore. Kar2p directly interacts with the translocating chain and uses the energy of ATP hydrolysis to pull the chain into the lumen acting as a translocation motor in a manner similar to that proposed for the action of

HSP70/TIM44 in mitochondrial protein translocation (Brodsky, 1996; Schatz *et al*., 1996). It remains to be determined whether Sec63p stimulates the ATPase activity of Kar2p.

In vitro reconstitution of translocation in microsomes derived from kar2 mutants shows that Kar2p is involved in both post-translational and co-translational translocation (Brodsky *et al*., 1995). Kar2p acts at an early stage involving interaction of the nascent polypeptide with the Sec61p complex, and by direct interaction with the nascent chain as it emerges into the lumen. (Sanders *et al*., 1992).

A general model was proposed : A polypeptide to be translocated post-translationally interacts first with the Sec63p complex and is then transferred to the Sec61p complex; Kar2p is required for either the interaction of the polypeptide with the Sec63p complex or the transfer of the polypeptide from the Sec63p complex to the Sec61p complex. (Figures 8 and 9).

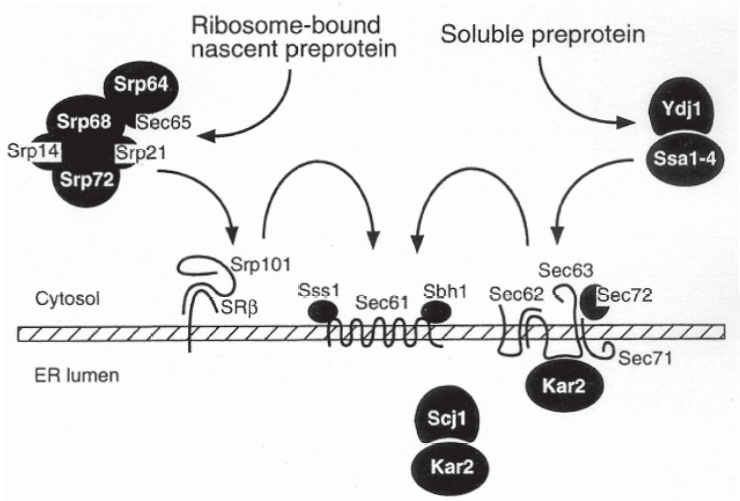


Fig.9. Soluble and membrane proteins involved in preprotein translocation accross the ER membrane. (Kaiser *et al*., 1997).

I.3.5. Signal peptidase

As secretory proteins enter the lumen of the ER, their signal sequences are proteolytically removed. The enzyme responsible for signal sequence cleavage is a luminal protein complex known as signal peptidase. Signal peptidase isolated from *S.cerevisiae* is composed of four polypeptides of 25, 20, 18, and 13 kD, and the corresponding genes have been identified (YaDeau *et al*., 1991; *Fang et al*., 1996; Mullins *et al*., 1996).

I.4. Quality control and maturation of protein in the ER

A variety of quality control mechanisms operate in the ER to ensure the fidelity and regulation of protein expression during cell life and differentiation. Quality control improves folding efficiency by retaining proteins in the special folding environment of the ER. Only proteins that pass a stringent selection process are transported to their target organelles and compartments; if proper maturation fails, the aberrant products are degraded, thus preventing harmful effects that could be caused by the deployment of incomplete folded or assembled proteins.

I.4.1. Protein retention and folding in the ER

The most commonly observed quality control mechanisms involves the association of newly synthesized proteins with ER folding enzymes and chaperones such as protein disulfide isomerase (PDI), calnexin, calreticulin, Erp57, and Erp72.

Secretory proteins often contain disulfide bonds that form when the proteins enter the oxidizing environment of the ER lumen. A link between disulfide bond formation and protein export from the ER is evident from experiments in which living yeast cells treated with the reducing agent DTT show an accumulation of CPY in the ER. CPY is a protein whose native form contains five disulfide bonds (Winther and Bredam, 1987).

Protein disulfide isomerase (PDI), which catalyses rearrangement of disulfide bonds, is essential for proper protein folding in the yeast ER. The amino acid sequence of the yeast

gene (*PDI1*) contains two CXXC active site motifs as well as a carboxy-terminal HDEL signal for retention in the ER (Scherens *et al*., 1991). Yeast carries two additional genes that appear to encode PDIs that are located in the ER. The sequences of *EUG1* and *MPD1* share some features with *PDI1*, and both genes suppress the lethality of a chromosomal deletion of *PDI1* when they are overexpressed, indicating that each is capable of carrying out the essential function of *PDI1* (Tachibana and Stevens, 1992; Tachikawa *et al*., 1995). *EUG1* contains two active site CXXS motifs and *MPD1* contains a single active site CXXC motif.

CNE1 was identified by its homology to calnexin, an abundant integral membrane protein of the mammalian ER. Calnexin and calreticulin, two homologous ER-resident lectins, bind to almost all soluble and membrane-bound glycoproteins that have monoglucosylated trimming intermediates of the N-linked core glycans. Together with Erp57, a thiol oxidoreductase, with wich they form complexes, they mediate retention and promote proper folding of glycoprotein substrates (for review, see Ellgaard *et al*., 1999). Cne1p is 24% identical to calnexin and both proteins have amino terminal luminal domains that bind calcium. Like calnexin, Cne1p may be important for the retention of incorrectly folded proteins in the ER (Parlati *et al*., 1995). How *CNE1* participates in ER retention is not known.

Eukaryotic cells face the additional challenges of coordinating sudden environmental changes and physiological stresses such as osmotic shock, nutrient availability, and heat shock, between different cellular compartments. In times of stresses in the ER, adaptative changes must be coordinated among protein folding capacity in the ER, transcription in the nucleus, and cytosolic synthesis of the proteins for the ER chaperones and protein processing enzymes. The pathway responsible for communicating changes in response to agents that compromise the proper assembly of proteins is called the **unfolded protein response** (UPR) (Shamu *et al*., 1994).

The regulation of *KAR2* by the unfolded protein response has been extensively investigated, and a 22-nucleotide segment known as the unfolded response element (UPRE) has been identified in the *KAR2* promoter that is necessary and sufficient for regulation (Kohno *et al*., 1993). Similarly, the genes for protein disulfide isomerases, *PDI1* and *EUG1*, show the

unfolded protein response and have promoter elements related to UPRE (Tachibana and Stevens, 1992). Mutations in *IRE1*, which encodes a transmembrane protein with an amino terminal luminal domain and a cytosolic domain that has homology with serine/threonine kinases, block the unfolded protein response (Cox *et al*., 1993; Mori *et al*., 1993).

In *S.cerevisiae*, the UPR is initiated when the N-terminal region of Ire1p senses the presence of unfolded proteins in the ER lumen, promoting Ire1p activation by autophosphorylation and oligomerization. Activated Ire1p allows the signal to be transmitted accros the ER membrane and functions as a site-specific endoribonuclease that excises a translation-inhibitory intron from the mRNA encoding the UPR-specific transcription factor Hac1p, converting an inefficiently translated inactive mRNA to an actively translated one. Hac1p binds to the UPRE of genes encoding chaperones and other targets of the UPR and activates them. (for review, see Niwa and Walter, 2000).

I.4.2. Protein degradation in the ER

An important insight into the mechanism of ER membrane protein turnover has come from studies of genes in the ubiquitin pathway. It was known for some time that incorrectly folded proteins in the ER membrane are recognized as substrates for ubiquitination and subsequently degradated by the proteasome. Evidence for such a degradation process has also been sought by examining the turnover of defective forms of luminal secretory proteins. Certain mutants of the soluble protease CPY and proteinase A enter the ER, become glycosylated, and are then degraded. Degradation of these mutant proteins occurs even if transport from the ER is blocked by a *sec18* mutation, implying that proteolysis occurs prior to exit from the ER (Finger *et al*., 1993). The involvement of the proteasome in this degradation process was tested. Remarkably, mutants of either proteasome subunits (*pre1, cim3, and cim5*) or mutants of an ubiquitin-conjugating enzyme (*ubc7*) greatly slowed degradation (Hiller *et al*., 1996). The mutant CPY was proposed to be delivered to the proteasome by retrograde translocation of the misfolded glycoprotein from the ER lumen to the cytosol, since a form of the mutant protein that was both glycosylated and ubiquitinated and that appeared to reside on the cytosolic face of the ER could be detected (Hiller *et al*., 1996). This result suggests that not

only the cytosolic loops of ER membrane proteins are subject to the ubiquitin/proteasome type of degradation, but that also soluble proteins in the ER lumen can be removed from the membrane, translocated from the lumen of the ER to the cytosol and then be degraded.

I.5. Precursors of GPI-anchored proteins

All described GPI-anchored proteins contain 2 hydrophobic amino acid stretches, one near the NH_2 terminus which serves as a signal sequence for import into the endoplasmic reticulum and another one at the COOH terminus which serves as a signal sequence for attachment to GPI. Both hydrophobic regions are removed rapidly from the newly synthesized protein (Conzelmann *et al*., 1987) and a preformed GPI is attached to the newly exposed COOH terminus by a putative GPI-protein transamidase (Hamburger *et al*., 1995). The GPI signal for attachment to GPI has four domains: the region containing the GPI attachment site (the ω site) plus the first and second amino acids downstream of the ω site (ω+1/ ω+2), a spacer of 5 to 10 hydrophilic amino acids, a hydrophobic stretch of 10 to 15 amino acids, and the short ω–minus region composed of five amino acids which is located upstream of the ω site (Nuoffer *et al*., 1993; Udenfriend *et al*., 1995; Hamada *et al*., 1998).

I.5.1. The ER signal sequence (see chapter III)

I.5.2. The GPI attachment region

I.5.2.1. The ω site

Comparison of known cleavage/attachment sites (called ω sites by Gerber *et al*., 1992) and mutagenesis studies on two mammalian proteins and one yeast protein (Micanovic *et al*., 1990; Moran *et al*., 1991; Nuoffer *et al*., 1993) have shown that only 5 or 6 amino acids with small side chains can act efficiently as ω sites. Mutagenesis of the ω site of placental alkaline phosphatase (Micanovic *et al*., 1990) showed that only 6 amino acids were suitable ω sites for GPI anchoring (D, G, A, C, S, and N) with N and S being approximatively equal and the best, followed by D, G, and A, which also showed about equal activity, followed by C. For decay

accelerating factor (DAF) (Moran *et al*., 1991), 5 amino acids (S, A, D, N, and G) gave relatively high levels of GPI-anchored DAF, while 2 amino acids (V and E) gave low, but detectable levels of GPI-anchored DAF. It was not possible to determine whether there are quantitative differences between the efficiences of the 5 amino acids that gave high levels of GPI-anchored DAF. The *S.cerevisiae* Gas1 protein is a typical GPI-anchored protein with a cleavable hydrophobic signal sequence for import into the ER and a COOH-terminal hydrophobic stretch (Nuoffer *et al*., 1991). Gas1p ω site (N^{506}) was then mutated to encode each of the 19 other amino acids. To determine which amino acid residues can act as a cleavage/attachment site, the mutant *gas1* alleles were subcloned into a centromeric vector and expressed from their natural promoter in a strain that carries a deletion in the *GAS1* gene. Cells were grown overnight, total proteins were extracted and analysed by Western blotting using antibodies raised against Gas1p. When the ω site is N (wild type), one detects almost exclusively the mature, 125-kDa fully glycosylated Gas1p and a very minor amount of a 105-kDa immature Gas1p species. When the *Gas1*-S^{506} and *Gas1*-G^{506} alleles are expressed, the pattern is similar with a small increase in the amount of the 105-kDa band. The *Gas1*-A^{506} allele leads to the expression of about equal amounts of 105- and 125-kDa Gas1p, whereas only a small fraction of the Gas1p encoded by the D^{506} and C^{506} alleles is in the 125-kDa form. All the other mutant gas1 alleles lead to almost exclusive synthesis of a 105-kDa species. This would suggest that only 6 amino acids at the ω site, N, S, G, A, D, and C, lead to substantial maturation of Gas1p. N, the wild type ω site was clearly the best while S and G were approximatively equal and clearly more effective ω site amino acids than A, D, and then C. From the analysis of the ω sites from human and yeast cells we can conclude that only amino acids with small side chains can act as suitable cleavage/attachment site.

Table 2. Amino acid preferences for the ω site.

Protein	decreasing preference
YGas1p	N > S, G > A, D > C
HPLAP	N, S > D, G, A > C
HDAF	S, A, D, N, G

I.5.2.2. The ω+1 and ω+2 sites

Analysis of the sequences downstream from the Gas1p ω site suggest that the ω+1 and ω+2 positions are also important. The ω+1 position of Gas1p can tolerate fairly large amino acids with only a small reduction of GPI anchoring efficiency. Even though small amino acids are preferred at the ω+1 site, they are not essential. Several mutations introduced at the position ω+1 and ω+2 of *Gas1*-G^{506} allele (this allele was used because mutations created downstream of the ω site show stronger phenotypes when the ω site is G than when it is N) show that amino acids with small side chain are preferred at position ω+1 and ω+2 even though the requirement is less strict for the ω+1 position. The combination of amino acids at the ω+1 and ω+2 positions may be crucial. Introduction of a relatively large amino acid at ω+2 position can be tolerated if the amino acid at ω+1 is very small. The opposite is also true (Nuoffer *et al*., 1993). This is entirely consistent with the results found from the analysis of the same sites on placental alkaline phosphatase (Gerber *et al*., 1992). To determine the amino acid requirements at the ω+1 and ω+2 positions, they used preprominiPLAP (see chapter VI) as substrate, generated by a rabbit reticulocyte translation system, and crude microsomal membranes from HeLa cells for processing. The prepro, pro, and GPI-linked forms were identified and assayed by an array of site specific precipitating antibodies, followed by SDS-PAGE. At the ω+1 site, of the 10 amino acids investigated, all yielded activity except proline. Substitution at the ω+2 site was extremely limited. Alanine and glycine were equally active, and traces of activity were observed with cystein and serine, whereas aspartate, threonine, glutamate, methionine, tryptophane, valine, histidine, and proline were totally inactive. Micanovic *et al*. (1990) and Kodukula *et al*. (1993) transiently transfected Cos-7 cells with mutant cDNAs and monitored the appearance of cell surface PLAP by immunocytochemistry as well as by enzyme activity released into the medium before and after treatment with PI-PLC. Of the amino acids tested at the ω+1 site, most yielded relatively large amounts of GPI-linked product, only the proline mutant was totally inactive. At the ω+2 site, the activity profile was: alanine=glycine> serine> threonine> aspartate> valine; glutamate, histidine, and proline were totally inactive.

Even though small amino acids are preferred at the ω+1 site, they are not essential. Indeed, in the case of human Thy-1 glutamate is found at this position naturally (Seki *et al*., 1985).

Both the ω site and ω+2 site amino acids influence the efficiency of GPI anchoring, the effects are cumulative, and proline is the only amino acid that exibits zero activity when present at any of the ω, ω+1, or ω+2 sites.

Table 3. Experimentally determined hierarchy of amino acids allowable at ω, ω+1, or ω+2 sites.

Mutant	ω	ω+1	ω+2
Ala	0.4+	1.0+*	1.0+*
Arg	ND	0.5	ND
Asn	0.8+	+	ND
Asp	0.4+*	0.4+	0.1
Cys	0.2+	0.3	0
Gln	0	ND	ND
Glu	0	+	0
Gly	0.4+	+	0.7+
His	ND	ND	0
Leu	0.1	ND	ND
Lys	0	ND	ND
Met	0	0.3	ND
Pro	0	0	0
Ser	1.0+	0.6+	0.3+
Thr	0	0.3+	0.1+
Trp	0	>0.1	(0)
Tyr	0	ND	ND
Val	0.1	ND	0.1

The data were obtained from Cos-7 cells transfected with wild-type and mutated forms of PLAP. The amino acid yielding the highest activity at each site was arbitrary given as value 1.0; all other values are relative to it. The parenthesis around the zero for tryptophan indicate that the experiment was carried out in cell free system. + indicates that this amino acid is present at the corresponding site of a characterized protein. * indicates the wild-type amino acid that is present at the respective position in PLAP. (Kodukula *et al*., 1993).

I.5.2.3. The spacer between the ω site and the COOH-hydrophobic domain

Moran and Caras (1991) suggested that the ω site must be to the NH2-terminal side and placed 10-12 amino acids from the hydrophobic domain. To test the effect of changes in the distance between the ωsite and the COOH-terminal hydrophobic domain on the GPI anchoring efficacity, mutants of Gas1p were created and their corresponding maturation measured (Nuoffer *et al*., 1993).

Addition of 1 amino acid (+T at position ω+3) inhibited GPI anchoring of Gas1p significantly while adding 2 amino acids (+TN at position ω+3) inhibited this process more strongly. Removal of 1 amino acid, be it the T or N at positions ω+3 or ω+4, respectively, caused a strong defect in GPI anchoring of Gas1p. Surprisingly, when both of these amino acids were removed, GPI anchoring of Gas1p was not inhibited as strongly as when either of the single amino acids were removed. These results suggest that, while a certain minimal and maximum distance may exist between the ω site and the COOH-terminal hydrophobic domain, it is not solely the number of residues that is important for the function of this region. Natural exemples of GPI-anchored proteins show that there is a large sequence diversity in this region . One possible explanation for the role of the spacer region comes from the hypothesis that the COOH-terminal hydrophobic domain and the ω and ω+2 sites bind to the putative transamidase. The region in between these two elements would have to be able to fold appropriately to allow these binding steps to occur. The lack of sequence conservation in this region would simply reflect the large number of possible ways to fold into an acceptable structure and could help to explain why the number of residues between the ω site and the COOH-terminal hydrophobic stretch is not tightly conserved.

I.5.2.4. The C-terminal hydrophobic domain

The function of the C-terminal hydrophobic domain was investigated, and several mutant forms of Gas1p, generated by site-directed mutagenesis, were analysed (Nuoffer *et al*., 1991). The mutant genes were expressed in the *gas1* null mutant, and the properties of the mutant proteins were analyzed by using the TX-114 phase separation system and Western blotting. A protein truncated at the C terminus, containing the anchor attachment site but lacking the

entire C-terminal hydrophobic domain, was secreted into the medium, indicating that it failed to be attached to the membrane by addition of the GPI anchor. Another mutation was constructed to assess the importance of the hydrophobic character of the C-terminal domain. A positive charge (L at position 526 replaced by R) was introduced into the hydrophobic domain; the mutant gene did not complement the growth defect of the *gas1* null mutant, and the modified protein was not anchored to the membrane but secreted into the medium. Introduction of this positive charge is sufficient to abolish anchor attachment. These results are consistent with data on mammalian proteins (Caras *et al*., 1989, 1987; Kurosaki *et al*., 1989; Lanier *et al*., 1989) suggesting that an essential determinant for GPI anchor addition is located in the C-terminal domain of the proteins; correct function of this determinant depends on the hydrophobic character of the sequence. Thus, even though the C-terminal domain is ultimately removed from the polypeptide, its presence in the primary translation product is essential for anchor addition.

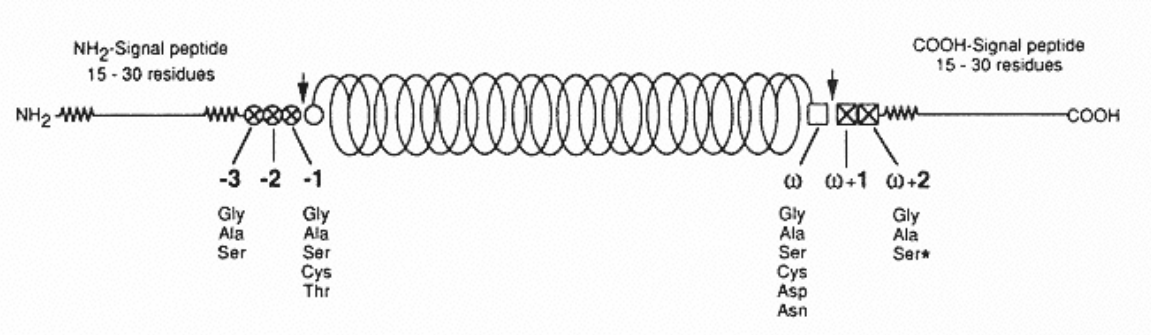


Fig.10. Structure of a typical nascent protein destined to be GPI anchored. Arrows point to sites of cleavage of the two signal peptides from the mature protein (helical structure). The solid lines in the signal peptides represent hydrophobic domains; jagged areas represent the hydrophilic domains, those nearest the cleavage sites being putative hinge regions. Amino acids shown at the –1 and –3 sites are those reported in most nescent proteins. Amino acids at the ω and ω+2 positions represent observations on characterized proteins and were verified experimentally. (Gerber *et al*., 1992).

I.6. The transamidation reaction

Observations on GPI addition in intact cells revealed that nascent proteins destined to be processed to a GPI form must have both NH2 and COOH terminal signal peptides, the first for translocation into the ER and the second for recognition by the enzyme(s) that catalyses condensation of the ethanolamine of GPI with the COOH at the ω site of a proprotein along with the elimination of the signal peptide. Fergusson and Williams (1988) investigated the kinetics of the reaction and showed that it is so rapid that the condensing and signal peptide elimination must be concomitant reactions as would be catalysed by a transamidase. Mayor *et al*. (1991) have shown that membrane fractions from trypanosomes incorporate GPI into VSG (condense the ethanolamine of GPI with the ω site of a pro-VSG) in the absence of an energy source and that the reaction is not stimulated by the addition of ATP or GTP. Production of an amide or peptide bond requires energy unless it is catalysed by a transamidase (Buchanan *et al*., 1973) or transpeptidase (Tate *et al*., 1974). Further credence to a transamidase nature of the enzyme-catalysed reaction comes from studies in cell free systems. By coupling crude microsomal membranes (RM), prepared from CHO or WISH cells to a rabbit reticulocyte lysate translation system, Bailey *et al*. (1989) and Micanovic *et al*. (1990) demonstrated the sequential conversion of the nascent protein, prepro placental alkaline phosphatase (preproPLAP), first to proPLAP then to mature GPI-linked PLAP. They identified the three products of cotranslational processing not only by their relative mobility on gels but also by their interaction with three site-specific, precipitating antibodies. However, PLAP, like most nascent proteins, are not ideal substrates for investigating the cell-free biosynthesis of a GPI protein. Changes of 2-3 kDa between precursors and products due to the loss of a signal peptide, are not easily discernible; for smaller proteins like Thy-1, changes in mass during processing are obscured due to high glycosylation. The problem was resolved by the design of a smaller and simpler engineered substrate for GPI processing derived by deletion of about two-thirds of the internal sequence of preproPLAP 513 (Kodukula *et al*., 1991). The engineered protein, preprominiPLAP 208, retains both signal peptides and all the epitopes for site-directed antibodies to PLAP but is devoid of glycosylation sites, the catalytic site, and most of the cystein residues (Figure 11). PreprominiPLAP markedly simplified studies in cell free conditions.

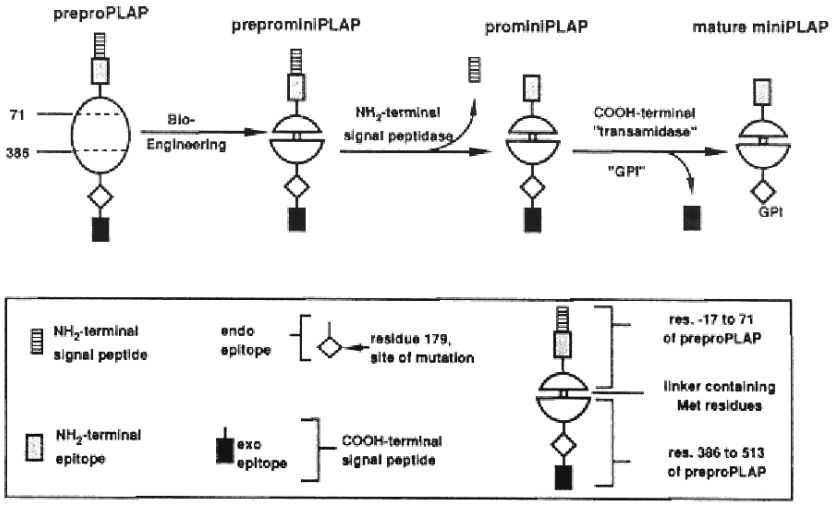


Fig.11. Structural relationship between prepromini PLAP and wild-type preproPLAP. (Kodukula *et al*., 1991).

Amthauer *et al*. (1992) used this cell-free, prepromini PLAP based system, and showed that while ATP increased conversion of prominiPLAP to GPI-linked miniPLAP in RM, the energy was required to release the chaperonin BiP and not for formation of the amide bond of the GPI protein. In their translation-independent cell free system, there was a small, but appreciable conversion of prominiPLAP to the GPI form, even in the absence of ATP and GTP, which indicates the presence of a pool of prominiPLAP that do not require an external source of energy for cleavage and GPI addition (as in Figure 12, *top, middle*). A comparable pool of the pro form of VSG in trypanosomal lysates would explain the findings of Mayor *et al*. (1991). Conceivably, ATP and/or GTP may be required for two steps in COOH-terminal processing: generation of GPI itself and translocation of prominiPLAP to the transamidase site in the ER. ATP may also be required for proper folding of the pro protein mediated by a chaperonin such as BiP (Rothman *et al*., 1989), and GTP may be required for translocation of this well folded pro protein to the transamidase site. (Figure 12, *top*).

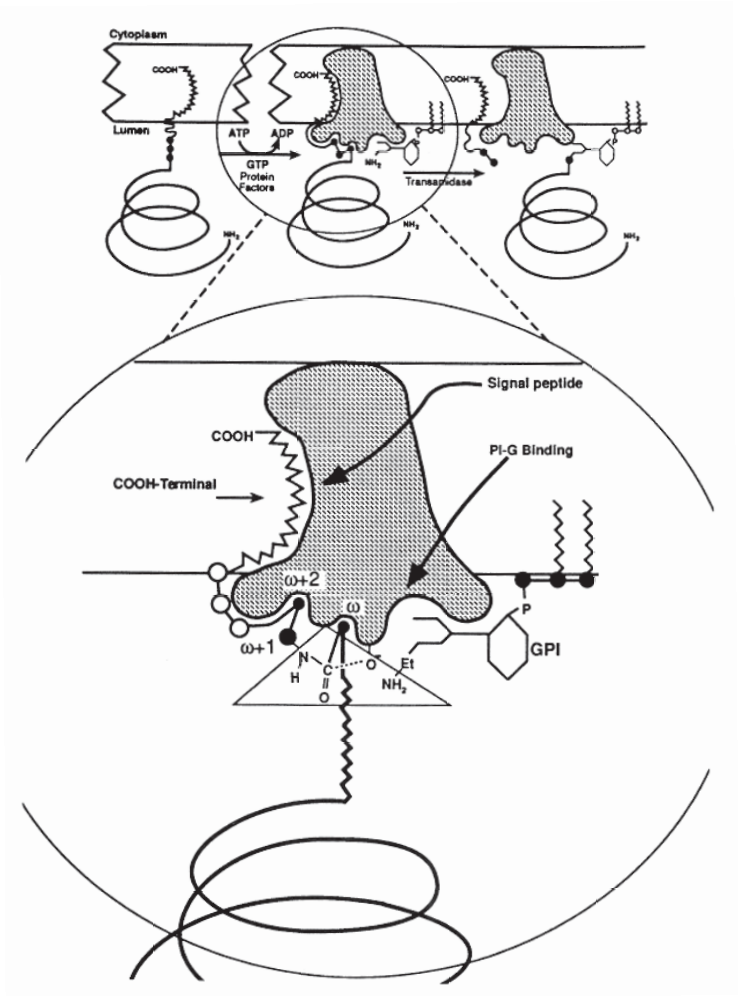


Figure 12. Amthauer *et al.* (1992) model for processing of prominiPLAP in the ER. *Top*. A first pool of prominiPLAP has its COOH-terminal signal peptide within the membrane but not in contact with the putative transamidase (top); a second and smaller pool of proprotein appears to be at the site of the transamidase (*top, middle*). This small pool of prominiPLAP does not require ATP or GTP for conversion to the GPI form because folding and translocation to the site of the transamidase have already taken place. *Bottom*. Detailed view of an intermediate Transamidase-proprotein-GPI complex presented by Gerber *et al*. (1992). This representation takes into account the features of the COOH-terminal signal peptide, including the hydrophobic sequence, the hinge region, and the small amino acid domain with selective binding sites for the ω and ω-2 residues. The carbonyl group of the ω residue of the proprotein is shown as being activated by a nearby hydrophilic oxygen (or SH group) on the

transamidase. This aspect of the model is based on known mechanisms of transamidation and transpeptidation. The binding site for the free GPI moiety on the transamidase is shown with the NH2 group of its ethanolamine residue in proximity to the activated carbonyl group of the proprotein, which enables nucleophilic attack to form the ethanolamide linkage accompagnied by cleavage of the signal peptide between the ω and ω+1 residues as shown in Figure 13.

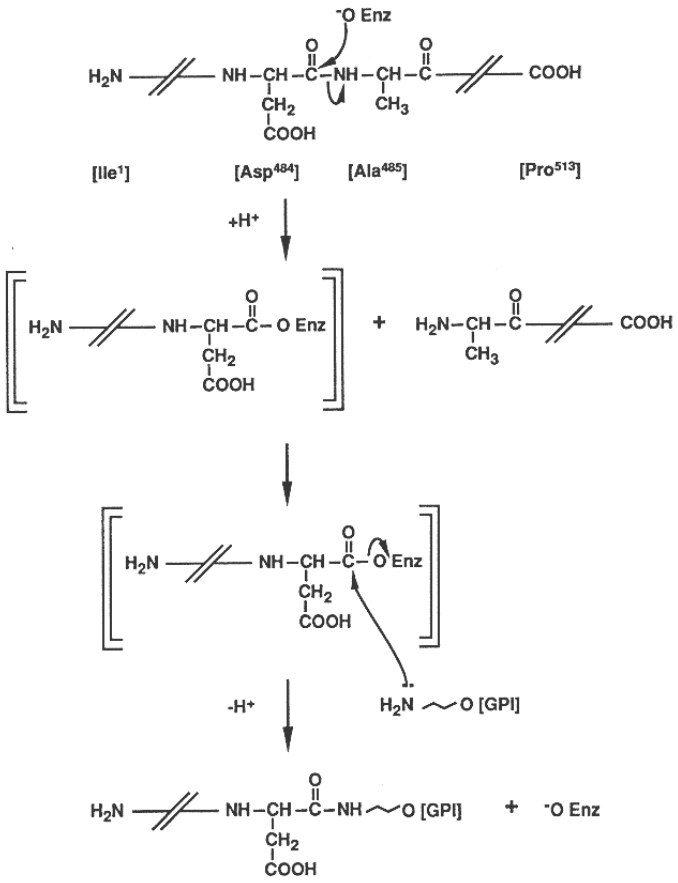


Fig.13. Proposed intermediate steps in the transamidation of proPLAP to GPI-linked PLAP.

More recent studies from Maxwell *et al*. (1995a&b) demonstrated that during the cell free processing of prominiPLAP in RM from GPI-deficient cells, while the major product was GPI linked, as much as 40% was cleaved at the correct ω site, but with the addition of the elements of water instead of GPI. The unusually large amounts of free miniPLAP formed

from the ω mutant (Ser in place of Asp), again only in the presence of GPI, lends further support to a transamidase-GPI-prominiPLAP complex. Of the six amino acids that can serve effectively at the ω site, only serine can form a six-membered ring as part of the complex. Initial enzymatic attack at the amide function between Ser179 and Ala180 (Figure14, *top*) can result in the formation of a partially stabilized six-membered intermediate (Fig14, *Middle*). Such an intermediate would undergo rapid interaction with the NH2 group of GPI to give the GPI-linked protein as the major product. However, the greater stability of the serine intermediate may permit appreciable time for hydrolysis to occur via pathway **b** (Fig14, *Bottom*) as a competing side reaction. When Ser179 is replaced by one of the other amino acids (which would not result in stabilized intermediates), this hydrolytic side reacton would be minimized and pathway **a** would predominate.

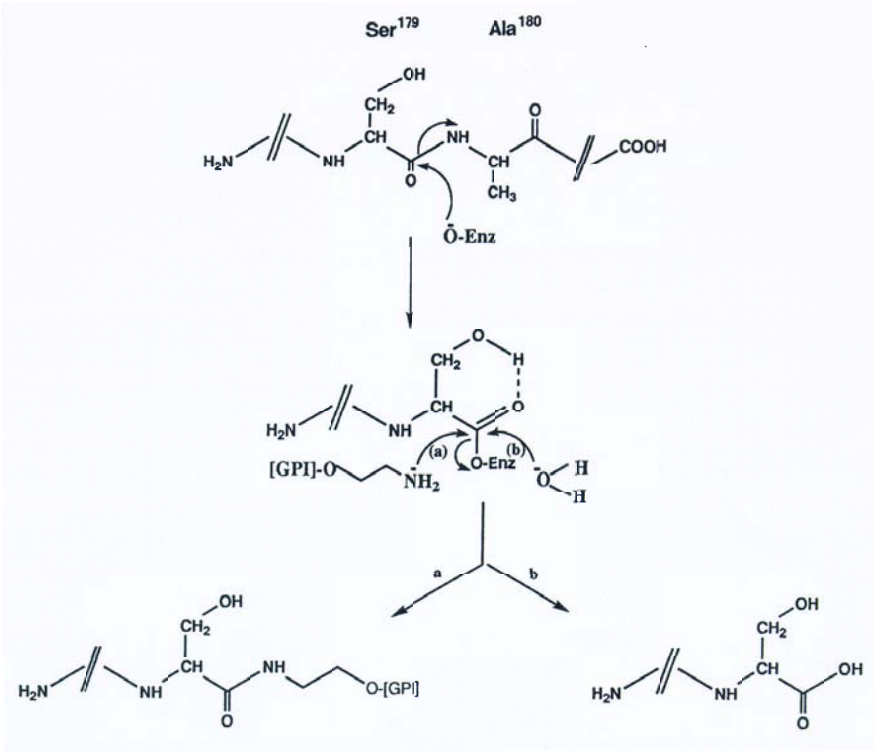


Fig.14. Proposed mechanism of cleavage of prominiPLAP-208 ωSer by the putative transamidase to yield both GPI-linked and free miniPLAP. (Maxwell *et al*., 1995a).

In a second experiment, Maxwell e*t al*. 1995b) showed that in the presence of rough microsomal membranes ,two small nucleophiles, hydrazine and hydroxylamine, catalyse in an enzyme dependent manner the conversion of prominiPLAP to mature forms from which the COOH-terminal signal peptide has been cleaved, possibly at the ω site but without the addition of GPI. The products of the processing of prominiPLAP in RM in the presence of different nucleophiles are summarized in Figure15.

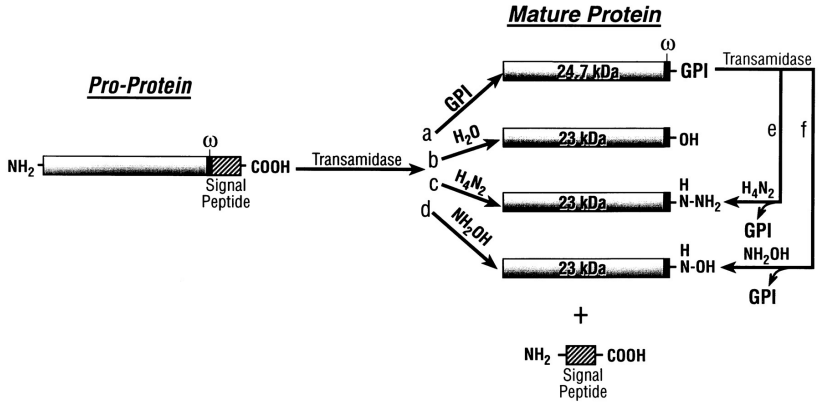


Fig 15. Summary of the processing of prominiPLAP in RM in the presence of different nucleophiles . (Maxwell *et al*., 1995b).

Both studies report enzyme-catalyzed cleavage of the signal peptide after activation of a carboxyl group in the absence of an energy source so that it can condense with an appropriate nucleophile; this is the hallmark of a transamidase.

I.7. The transamidase complex

I.7.1 Gpi8p

I.7.1.1. Cloning of *GPI8*

The biosynthesis and intracellular transport of two GPI proteins, namely Gas1p and α-agglutinin, have been studied in some detail (Frankhauser *et al*., 1991; Lu *et al*., 1994).

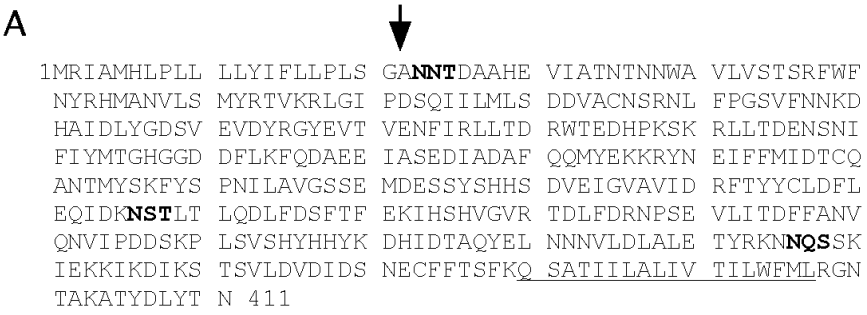
Proteins receive their anchor in the ER, and are transported to the Golgi apparatus by vesicular traffic (Conzelman *et al*., 1988; Schekman, 1992). Whereas Gas1p is destined to stay at the plasma membrane (Nuoffer *et al*., 1991), α-agglutinin loses the inositolphospholipid moiety after having reached the plasma membrane, and appears at the outer surface of the cell wall (Lu *et al*., 1994). The mature α-agglutinin becomes covalently linked to the β-glucans of the cell wall (Lu *et al*., 1995). α-agglutinin has been shown to critically depend on its GPI anchor for integration into the cell wall, since removal of the COOH-terminal hydrophobic GPI signal sequence resulted in the loss of cell surface attachment, and allowed efficient secretion of the truncated α-agglutinin (Wojciechowicz *et al*., 1993). Based on the assumption that mutants deficient in the biosynthesis of the GPI glycolipid or its transfer to proteins would be deficient in the expression of α-agglutinin at the cell surface, Benghezal *et al*. (1995) developed a new approach for the selection of GPI-anchoring mutants in yeast. The procedure selected cells that do not express α-agglutinin, a GPI protein, at the cell surface.

Strain W303-1B was mutagenized with ethyl methanesulfonate, and surviving cells were allowed to recover through six mitotic cell cycles at 24°C. Mutagenized cells where then incubated with a-factor to stimulate the expression of α-agglutinin, and the cells displaying a normal, constitutive expression of α-agglutinin were removed by immunoselection; cells were incubated with anti-α-agglutinin antibodies and absorbed onto anti-IgG-coated magnetic beads. The negatively selected remaining cells, assumed to be unable to express α-agglutinin at 37°C, were plated for growth at 24°C. Of the growing clones, those that did not grow at 37°C were selected, and these *ts* clones were screened for acid phosphatase secretion at 37°C in order to eliminate mutants deficient in transcription, translation, translocation into the ER, and secretion. Clones which secreted acid phosphatase normally were labeled with 2-^{3}H Inositol and screened for the presence of abnormal labeled lipids. 10 isolated mutants accumulated distinct abnormal lipids. For one of these mutants, so called *gpi8* , it was shown that the accumulated abnormal lipid corresponded to the complete GPI precursor. The *GPI8* gene was cloned by complementation of the temperature sensitive phenotype of the *gpi8-1 gpi7-1* double mutant (Benghezal *et al*., 1996).

The *gpi7-1* mutant is one of the 10 GPI-anchoring mutants selected above and was shown to accumulate a lipid corresponding to a complete precursor lacking a polar phosphoethanolamine substituent. *gpi8-1* and *gpi7-1* mutants still grow at 37°C, albeit more slowly. The double mutant *gpi8-1 gpi7-1* grows well at 24°C but not at 37°C, facilitating the selection of complementing genes. After transformation with a genomic yeast library, colonies were tested for growth at 37°C, plasmids were extracted, amplified and tested for their ability to suppress the abnormal GPI lipid accumulation of single mutants *gpi8-1* or *gpi7-1*. The plasmid complementing *gpi8-1* contained a fragment of 2.4 kb and was termed YEp*GPI8*. The sequence, essential for viability, contained one open reading frame mapping to chromosome IV and predicting 411 amino acids for a protein of 47 kDa.

The hydrophilicity plot according to Kyte and Doolitle reveals two hydrophobic regions: one at the N-terminus qualified as a signal peptide for translocation into the ER (von Heijne, 1986), and a second one localized at the C terminus and predicted to be a transmembrane helix (Rost *et al*., 1995). Antibodies against a soluble N terminal fragment of Gpi8p react with four bands of 50, 48, 46, and 44 kDa indicating different states of glycosylation. Upon treatment of the membrane bound 46-50 kDa forms of Gpi8p with endoglycosidase H, Gpi8p migrates as one single band of 44 kDa, indicating that Gpi8p contains three *N* -linked oligosaccharides. The membrane topology of Gpi8p was investigated by a protease protection assay and demonstrates that the bulk of Gpi8p is lumenal. The cellular localisation was determined by subcellular fractionation and Gpi8p was found to co-fractionate with Wbp1p, a subunit of the oligosaccharyltrabsferase which resides in the ER (te Heesen *et al*., 1991). In contrast with Wbp1p, Gpi8p does not contain any known retrieval sequence. These results demonstrates that Gpi8p is a glycosylated transmembrane protein of the ER.

A



B

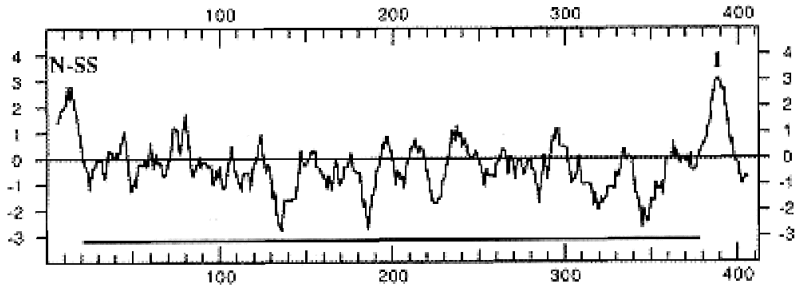


Fig.16. Sequence analysis of Gpi8p. (A) N-glycosylation sites are in bold face, the C-terminal transmembrane domain is underlined. The most likely cleavage site of the signal peptidase is indicated by a vertical arrow. (B) The hydropathy plot according to Kyte and Doolittle predicts two transmembrane domains: one at the N-terminus being the ER signal sequence, and another C-terminal domain (1) supposed to be the ER retention signal.

I.7.1.2. Gpi8p active site

Data base searches revealed significant homology (28-28%) between Gpi8p and a family of cysteine proteinases found in plant seeds or invertebrates and classified as cysteine proteinase family C13 in the SWISSPROT database. Plant proteases are Asn-specific endopeptidases and have been isolated from mature castor beans (Hara-Nishimura *et al*., 1991), soybeans (Muramatsu and Fukasawa, 1993), arabidopsis thaliana (Kinoshita *et al*., 1995) and jack beans (Abe *et al*., 1993) and are involved in the proteolytic processing of proteins in the protein-storage vacuoles. It has been reported that concanavalin A, the commonly used jack bean lectin, is produced by the post-translational proteolysis and transpeptidation of the COOH of Asn281 residue close to the C terminus. After removal of the signal peptide (1-29), the last 9 amino acids at the C terminus of the precursor protein are removed and the α-amino group of Ser30 is attached to the liberated COOH of

Asn281, a process which creates a circular peptide. Formally this maturation step is analogous to the transamidation postulated for the addition of GPIs to proteins, whereby in GPI anchoring it is not the N terminal end of the peptide but a GPI which steps in to replace the original C-terminal peptide.

The study of cysteine proteases showed that many of them contain a catalytic triad (Asn, His, and Cys) like papain, or at least a catalytic diad (His and Cys) like caspases. His helps to deprotonate the active site Cys in the same way as is described for the active site Ser of serine proteases (Blow and Steitz, 1970; Sprang *et al*., 1988). Meyer *et al*. (1999) showed that mutation of the conserved Cys199 of Gpi8p to Ala completely abolished the activity of the protein. Mutation of the only other conserved Cys of the Gpi8 subfamily, Cys85, does not abolish Gpi8p function. Finally, Cys373 is not required for catalytic activity (Fraering *et al*., in preparation). Mutation of the conserved His157 also completely abolishes the functionality of Gpi8p, whereas mutation of the only other conserved His of the C13 family, His54, only leads to partial loss of function (Meyer *et al*., 1999). It therefore is likely that deprotonation of Cys199 is mediated by His157 and not by His54. The replacement of a stretch of 6 amino acids upstream of Ser60 by a consensus sequence elaborated from all members of the C13 protease family (Ser60->Cys59 allele) is completely unable to rescue Δ*gpi8*, suggesting that this region, including Ser60 is important for the catalytic activity of yGpi8p (Meyer *et al*., 1999). All these findings suggest that, the Cyst and His residues corresponding to Cyst199 and His157 of yGpi8p represent the active site residues, and that the region around Ser60 plays some important role.

Ala substitutions of Cys206 and His164 in human Gpi8p, residues that are conserved in yeast and nematode Gpi8ps, resulted in complete loss of complementation of class K cells (a human mutant cell line with a defect in the GPI8 gene), whereas substitution of Cys92 only partially decreased the activity. Ser67 was not important. (Ohishi *et al*., 2000). Yeast Gpi8p has no activity when transfected into class K cells.

Hilley *et al*. (2000) cloned the *Leishmania mexicana* GPI8, and showed the high level of sequence identity existing among the predicted yeast, human and *L.mexicana* Gpi8 proteins, including the conservation of cysteine and histidine residues involved in the catalytic mechanism.

I.7.1.3. Catalytic sites of cysteine endopeptidases

It was found that a motif **His**-Gly-spacer-Ala-**Cys** can be recognised in a clan called CD comprising the families of Gpi8p (C13), caspase-1 (C14), clostripain (C11), and gingipain R (C25) (Chen *et al*., 1998). The caspases are mammalian cytosolic endopeptidases that play key roles in apoptosis, whereas clostripain and gingipain are cystein endopeptidases from the pathogenic bacteria Clostridium histolyticum and Porphyromonas gingivalis, respectively (Porter *et al*., 1971; Potempa *et al*., 1998). Alignements of the catalytic sites of cysteine endopeptidases of each family are shown in the Figure 17 and reveal in all four families a block of four hydrophobic amino acids (marked in green in Figure17) closely preceding each of the catalytic residues. The three dimensional structure analysis of gingipain R (RgpB), determined at high resolution by x-ray crystallography, confirms not only some equivalency, but indicates considerable similarities regarding polypeptide fold and active site geometry (Eichinger *et al*., 1999). Furthermore, it suggests closer structural similarities of gingipain with legumains and clostripains as well.

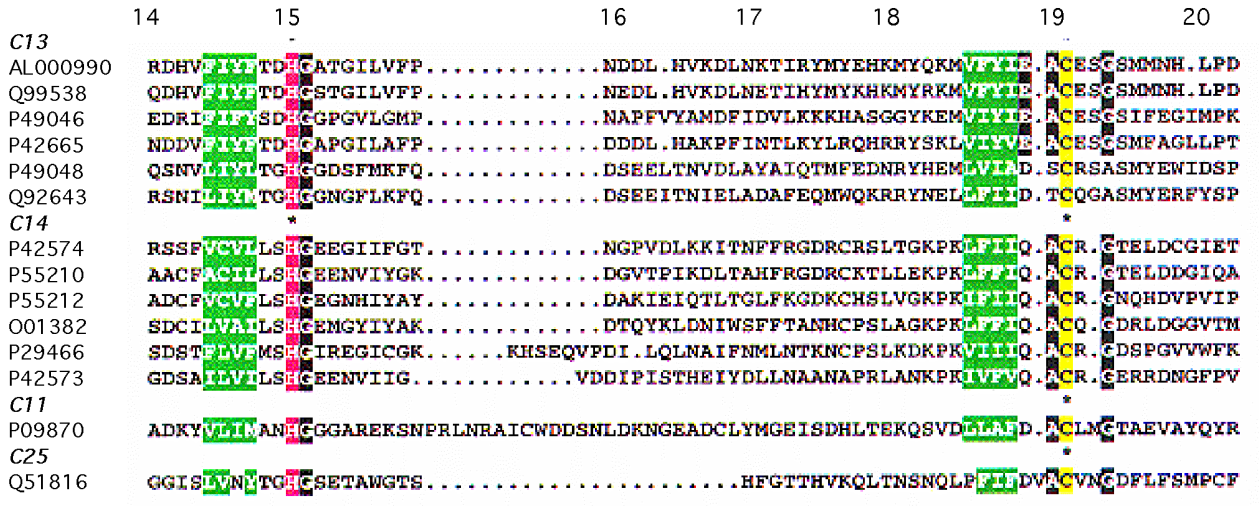


Fig.17. Alignement of the catalytic sites of cysteine endopeptidase of families of legumain (C13), caspase-1 (C14), clostripain (C11), and gingipain (C25). The amino - acid sequence segments containing the known or putative catalytic residues (magenta and

yellow) in each of the four families were aligned manually by the introduction of gap residues. Two or three residues N-terminal to each of the catalytic residues, is a block of four predominantly hydrophobic residues (green). Other residues that tend to be conserved are printed in white on black. Asterisks are placed under the His and Cys residues in each family for which they have been identified experimentally as catalytic. AL000990, mouse legumain; Q99538, human legumain; P49046, jack bean (Canavalia ensiformis) asparaginyl endopeptidase; P42665, Schistosoma japanicum haemoglobinase; P49048, C.elegans protein T05E11.6; Q92643, human GPI8 protein; P42574, human caspase-3; P55210, human caspase-7; P55212, human caspase-6; O01382, Drosophila melanogaster caspase; P29466, human caspase-1; P42573, C.elegans CED3 protein; P09870, Clostridium histolyticum α-clostripain; Q51816, Porphyromonas gingivalis gingipain R. Derived from (Chen *et al*., 1998).

I.7.1.4. Three dimensional structure analysis of gingipain R (RgpB).

According the observation that no closer homologues to human or yeast Gpi8p have been crystallized, the 3D structure of RgpB can serve as a model to deduce a putative 3D structure of the active site of the corresponding proteins.

I.7.1.4.1. Overall structure

As represented in Figure 18, the 2.16 A° resolution structure of RgpB revealed that the crown formed by the N-terminal 351 residues represents the catalytic domain, while the root made by the last 84 residues resembles an immunoglobulin superfamily (IgSF) domain. RgpB is characterized by a high ratio of regular secondary structure elements, with the catalytic domain exhibiting the structural motif of a typical α/β portein and the IgSF domain having an all-β conformation.

The catalytic domain can be subdivided into A- and B- subdomains. Each subdomain comprises a central β-sheet and a few additional hairpins flanked by helices on either side, as characteristic for α/β open-sheet structures. As usual in such open β-sheet enzymes, the active center is in a crevice outside the carboxyl end of the β-sheet, with the active-site residues His 211 and Cys 244 presented by loops s7-s7' and s8-h8.

Fig.18. Ribbon plot of RgpB (front view). The molecule consists of the catalytic domain (top) subdivided into A- (right) and B-subdomains (left), and the IgSF domain (bottom). Strands are shown as yellow arrows, helices as red spirals and the connecting segments as blue ropes. The catalytic residues on top are shown as orange stick models and the three putative calcium ions as golden spheres. The figure was prepared with MOLSCRIPT. (Eichinger *et al*., 1999).

I.7.1.4.2. Active site

Figures 19 and 20 allow views into and toward the active center of RgpB. The active site and its immediate environnement placed on the almost flat “mastivating surface” of the crown is demarcated by strand His 211-Glu 214 (Figure 19, bottom right), the rising segment Ala 243-Val 245 (right, center), the parallel aligned long s9-h9 connecting segment (top) and the perpendicular running twisted s5-h5 (left, from Glu 152 to Asp 163). In the center resides the exposed Cys 244, and from the bottom strand ejects the His 211 imidazole side chain, with its Nδ1 atom in all RgpB structures placed 5.5 A° away from the Cys 244 Sγ.

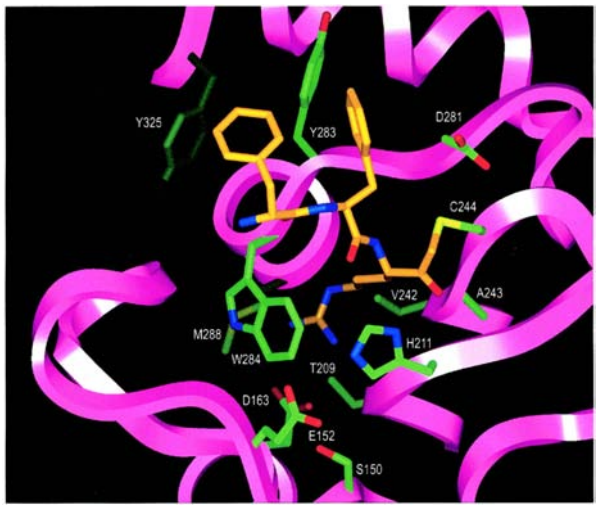


Fig 19. Interaction of the D-Phe-L-Phe-L-Arg methylene inhibitor with the RgpB active-site. The active-site region of RgpB, besides a few important residues (green) mainly represented by the ribbon-like backbone (pink), is shown in standard orientation (obtained from the front view, Figure 18, upon a 90° rotation about a horizontal axis). The inhibitor chain (yellow stick model) covalently linked via its methylene group to Cys244 Sγ (center, right) runs from left to right, with its Arg-P1 side chain reaching back into the S1 pocket. The imidazole side chain of His211 and the carboxylate of Glu152 are arranged on the molecular surface (bottom) opposite to Cys244. The figure was prepared with Insight II. (Eichinger *et al*., 1999).

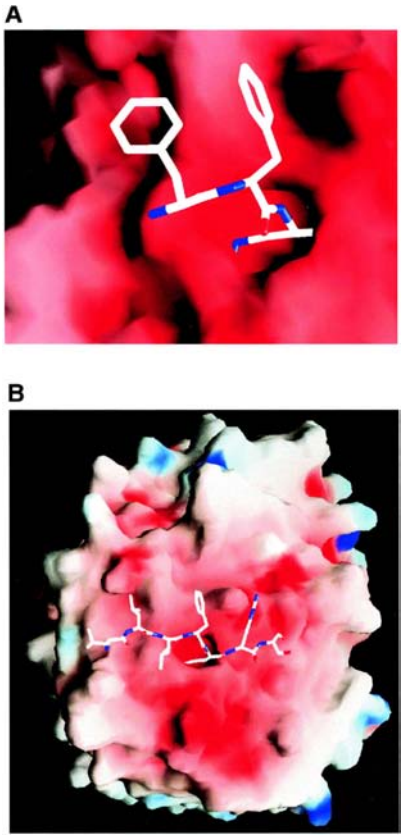


Fig.20. View toward the solid surface of the RgpB active-site (standard orientation). The electrostatic surface potentials are contoured from –15 (intense red) to 15 K_BT/e (intense blue). Figures were prepared with GRASP. (A) Close-up view toward the active center of the inhibited RgpB, with the D-Phe-L-Phe-L-Arg methylene moiety (stick model) covalently bound. (B) View toward the active-site of RgpB, with the modelled heptapeptide non-covalently attached (stick model) (Eichinger *et al*., 1999).

I.7.1.4.3. The catalytic mechanism.

The carbonyl group of the scissile Arg-Xaa peptide bond of a bound substrate is presented in a rigid and stereochemically favourable manner to Cys 244 for nucleophilic attack by Sγ (Figure 21). Assisted by the polarization of the P1 carbonyl in the oxyanion hole, Cys 244 Sγ could bind to the carbonyl carbon of the Arg-Xaa scissile peptide bond toward its Re face, under approach of the tetrahedral intermediate state. In this reaction, the attacking Sγ lone pair orbital might be oriented toward the carbonyl by hydrogen bonding from the Gln 282 N-H. Simultaneously, the imidazole group of His 211 positioned on the opposite side of the scissile bond could, possibly, in a concerted move together with the Glu 152

carboxylate group, turn toward the pyramidalizing Xaa leaving group nitrogen. The strongly negative electrostatic surface potential together with the properly placed Glu 152 would probably stabilize this His 211 imidazole in its protonated form, enabling it to donate a proton to the leaving group nitrogen, thereby promoting the C-N break in the bound substrate and the release of the C-terminal fragment (see Figure 21). The thiol ester remaining after release of the C-terminal fragment could be hydrolysed by a water molecule attacking the ester carbonyl and cleaving the ester bond, leading to the release of the N-terminal portion of the substrate under simultaneous transfer of both protons to the His 211 imidazole and the Cys 244 Sγ , respectively.

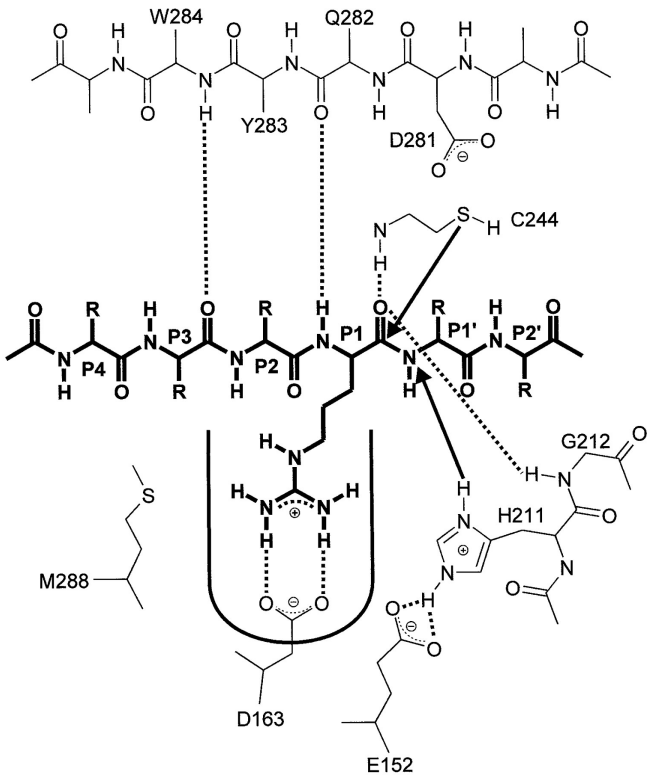
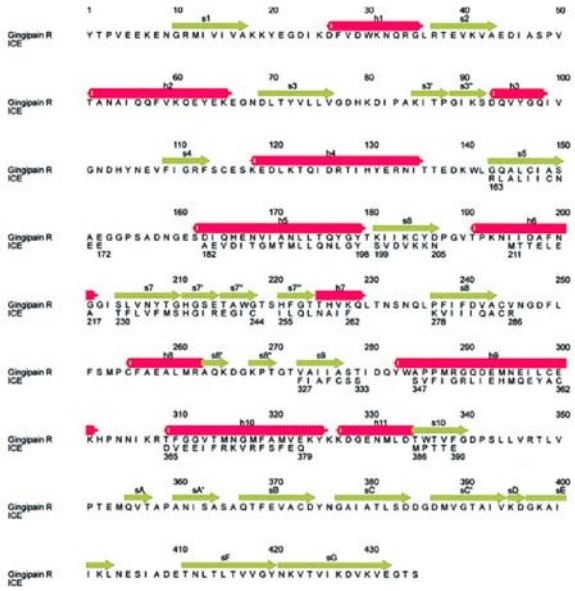


Fig.21. Schematic drawing of the probable peptide substrate-active centre interaction of RgpB deduced from FFRCMK. The view is in the standard orientation, so that the modelled substrate (thick connections) runs from left to right. Probable intermolecular hydrogen bonds are shown by dashed lines, while the routes of attack of the Cys244 Sγ on the Arg-P1 carbonyl and transfer of the His211 Nδ hydrogen toward the leaving group are indicated by arrows. (Eichinger *et al*., 1999).

I.7.1.4.4. Comparison with caspases.

A topological search of the RgpB structure performed with TOP3D (Lu, 1996) against all protein structures currently in the PDB revealed caspase-1 (Walker *et al*., 1994) and caspase-3 (Rotonda *et al*., 1996) as the only topological homologues (Figure 22). A closer look showed that the A- as well as the B-subdomain each contain a caspase folding motif corresponding to the p20-p10 heterodimer of caspase-1 and the p17-p12 heterodimer of caspase-3. After optimal superposition of gingipain's B-subdomain over the heterodimer of caspase-1, strands and helices s5, h5, s6, h6, s7, h7, and s8 of RgpB superimpose well with the first four strands and the first three helices of the caspase-1 p20 chain, with conserved strand order and direction (Figure 22A). The topological equivalency of the RgpB A-subdomain is less striking. According to the superposition of the RgpB B-subdomain on the caspase-1 heterodimer (data not shown) , both the catalytic Cys residues (211 and 237 respectively) occupy identical sites and exhibit very similar conformations. The similarity of the RgpB catalytic domain with caspases is certainly not just accidental. The identical arrangement of the secondary structure elements together with an almost identical active-center topology are a quite significant indicator of a close evolutionary relationship, suggesting that the usually selected (red-dark blue, Figure 22B) caspase heterodimer is indeed generated from a single pro-caspase chain (Thornberry and Lazebnik, 1998).

A



B

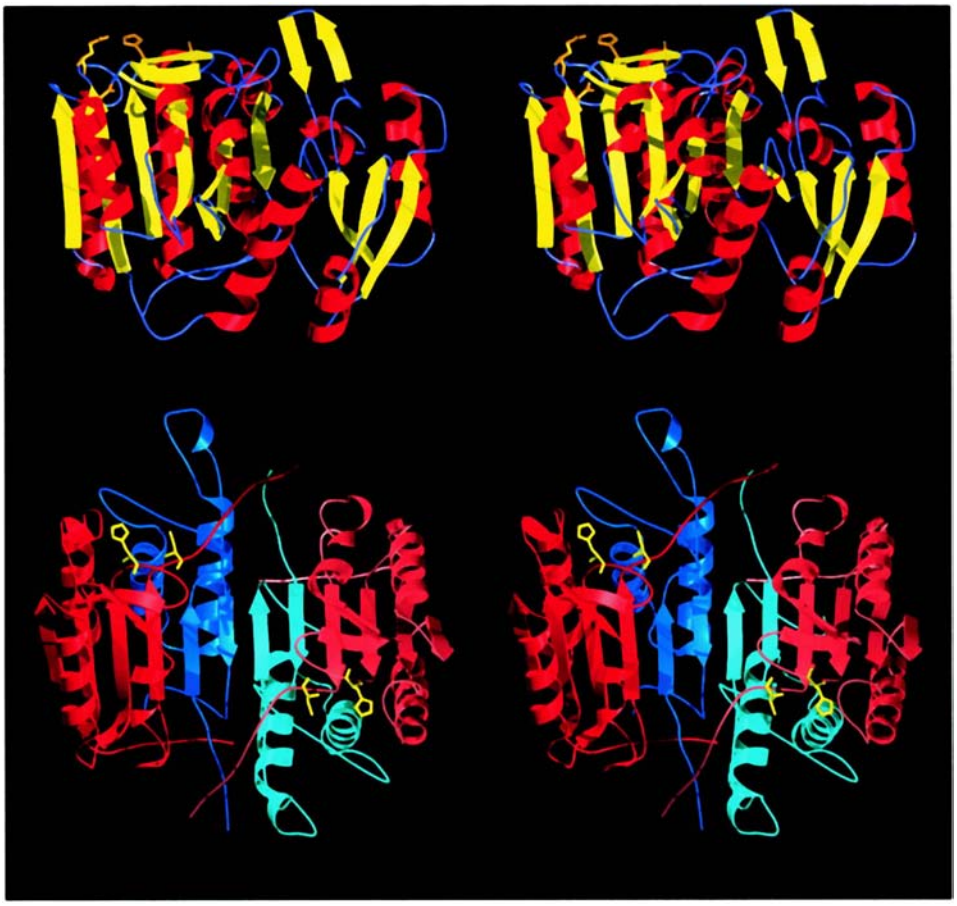


Fig.22. (A)Topological similarity between RgpB and caspase-1. Arrows and braces indicate β-strands and helices in RgpB. A total of 118 residues of caspase-1 have been aligned to the B-subdomain according to topological equivalency with caspase-1. The numbering is for RgpB (top) and caspase-1 (bottom). **(B). Comparison of the RgpB catalytic domain (top, in stereo, red, yellow and blue) and the caspase-3 tetramer (bottom, in stereo, with both p17 and p12 peptides given in red and blue.** The catalytic residues are shown as orange and yellow stick models. The caspase is presented in such an orientation that its left-side p17-p12 heterodimer half superimposes with the active RgpB B-subdomain. (Eichinger *et al*., 1999).

I.7.1.5. The lumenal region of Gpi8p is sufficient for its activity

Sequence comparison performed by Hilley *et al*. (2000) suggest that the *L. mexicana* Gpi8p is smaller than the *S.cerevisiae* and human homologues (38 vs. 46 kDa) and that it lacks the C-terminal hydrophobic domain that is thought to be involved in anchoring these other Gpi8ps to the lumenal leaflet of the ER (Benghezal *et al*., 1996; Yu *et al*., 1997). Human Gpi8p mutant bearing 321 amino acids and lacking the transmembrane domain was shown to retain its activity to complement class K mutant cells, indicating that the transmembrane domain is not necessary. (Ohishi *et al*., 2000). A mutant bearing 310 amino acids did not have any activity, indicating that the region from amino acids 311-321 is critical. Attachment to the ER membrane of the functional human Gpi8p mutant lacking the transmembrane domain, like *L.mexicana* Gpi8p, where the protein is able to interact with the preformed GPI anchor, may thus require one or more other integral membrane proteins. A possible candidate is Gaa1p, an integral membrane protein that appears to be an essential component of the GPI-transamidase complex of yeast (Hamburger *et al*., 1995).

I.7.2. Gaa1p

I.7.2.1. Cloning of GAA1

The *GAA1* gene was cloned by Hamburger *et al*. (1995) in *S.cerevisiae* by complementation of the temperature sensitive phenotype of a mutant described by Chvatchko *et al*. (1986); they reported the isolation of a yeast mutant, *end2* that is defective in the accumulation of an endocytic marker, Lucifer yellow, in the vacuole. To determine where in the endocytic pathway the mutant was affected, several analyses were performed. The ability of mutant cells to internalize and degrade α-factor, a marker of receptor-mediated endocytosis (Dulié *et al*., 1991) was analysed. No defect in pheromon internalisation was detected, suggesting that the effect of the mutation on the endocytic pathway is indirect. Additional experiments were performed in order to examine whether other membrane trafficking events were disturbed by the indirect effect of the mutation. The secretion of invertase was followed as a marker for the secretory pathway. The mature

core glycosylated invertase was found in both mutant and wild-type spheroplasts, indicating that the secretory pathway was not affected by the mutation. The ability of mutant cells to mature a GPI-anchored protein, Gas1p, was tested. Experiments performed after a 30-min incubation at 37°C showed that Gas1p maturation was completely blocked whereas the biogenesis of the vacuolar enzyme, CPY, was identical in mutant and wild type cells. To test for a defect in GPI anchoring, incorporation of 3H-inositol into proteins was analysed. The observed reduced incorporation of inositol in proteins suggested that the primary defect in the mutant cells was not in endocytosis, but in GPI anchoring; the *END2* gene was then renamed *GAA1* for GPI anchor attachment. In order to identify the mutated gene, the thermosensitive mutant cells were transformed with a plasmid DNA library and colonies showing plamid-dependent growth at 37°C were selected. Plasmids harboring a complementing activity were isolated from selected clones and the *GAA1* gene was sequenced.

I.7.2.2. GAA1 sequence analysis

The single, large GAA1 open reading frame encodes a protein of 68 kDa that shows no homologies with other known proteins. After generation of a heterozygous diploid by integrative transformation, sporulation, and tetrad dissection, it was found that the *gaa1* disrupted cells did not grow, indicating that the *GAA1* gene is essential. Gaa1p contains six regions of hydrophobic and/or uncharged amino acids that could span a lipid bilayer. Rabbit antibodies against the NH_2 and COOH-termini are both able to recognize a protein of 70 kDa, indicating that there is no removal of the ER signal sequence and that the protein is glycosylated. The glycosylation was confirmed by an endoglycosidase H treatment of Gaa1 protein that shifted the apparent molecular mass from 70 to 68 kDa. Specific analysis showed that only one glycosylation site (N383) was used. At the extreme COOH terminus of the protein there is a KXKXX sequence that has been characterized as a signal for retrieval of membrane proteins to the ER (Gaynor *et al*., 1994). To determine whether Gaa1p is localized in the ER, the protein was overexpressed and localized by immunofluorescence using the antibodies raised against the COOH terminus.

A

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1 MALLEKLHRR IVDMGLVPRI IALLPVISML CALFGFISIA ILPMDGQYRR
  TYISENALMP SQAYSYFRES EWNILRGYRS QIKEMVNMTS MERNNLMGSW
  LQEFGTKTAI YENEQYGETL YGVMHAPRGD GTEAMVLAVP WFNSDDEFNI
  GGAALGVSLA RFFSRWPVWS KNIIVVFSEN PRAALRSWVE AYHTSLDLTG
  GSIEAAVVLD YSSTEDFFEY VEISYDGLNG ELPNLDLVNI AISITEHEGM
  KVSLHGLPSD QLTNNNFWSR LKILCLGIRD WALSGVKKPH GNEAFSGWRI
  QSVTLKAHGN SGHDITTFGR IPEAMFRSIN NLLEKFHQSF FFYLLLAPRQ
  FVSISSYLPS AVALSIAFAI SSLNAFINNA YANISLFSEY NLVALLVWFV
  SLVISFVVSQ AFLLIPSSGL LMTISMASCF LPLILSRKIH ISEPLSYRLK
  NVAFLYFSLV STSLLMINFA MALLIGTLAF PMTFVKTIVE SSSEHEVTTQ
  SSNPIKTEPK DEIELVENHM DTTPATPQQQ KQKLKNLVLL ILTNPFISIT
  LFGLFFDDEF HGFDIINKLV SAWLDLKCWS WFVLCIGWLP CWLLILASSF
  ESKSVVVRSK EKQS 614
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B

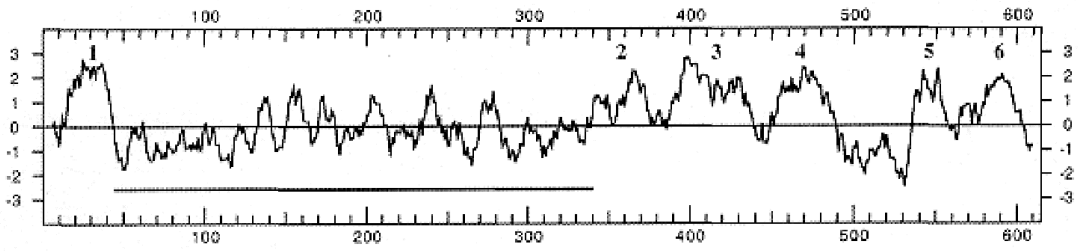

Fig.23. Sequence analysis of Gaa1p. (A) N-glycosylation consensus sites are in bold face, potential transmembrane domains are underlined, and the putative ER localization sequence is double underlined.(B) The hydropathy plot according to Kyte and Doolittle predicts six transmembrane domains (TMDs; from 1 to 6). Between the first and second TMD, a large hydrophilic ER-luminal domain is predicted (bar).

To define where the defect in GPI anchor attachment of the *gaa1* mutant lies, the biosynthesis of the GPI anchor was examined after labeling the cells with 3H-mannose and 3H-inositol. Lipids extracted from the *gaa1* mutant and separated by TLC showed a band comigrating with CP2, whereas in wild-type cells, this band was not detectable. The band corresponds to complete precursor as verified by several chemical and enzymatic tests. Thus, at restrictive temperature, the *gaa1* mutant cells were capable of synthesizing the entire GPI anchor precursor, but did not attach it to proteins, consistent with Gaa1p being part of the putative GPI transamidase. It is also possible to reason that Gaa1p acts to flip the complete GPI precursor to the lumen of the ER, thereby increasing the effective GPI concentration and that this may also improve anchoring efficiency.

Equivalent results were obtained in GAA1-knockout F9 embryonal carcinoma cells which were shown to loose the surface expression of GPI anchored proteins like Thy-1, stem cell

antigen-1 or heat stable antigen and to accumulate mature forms of GPI (Ohishi *et al*., 2000). Human and mouse GAA1 cDNAs are able to restore the expression of the GPI-anchored proteins in the GAA1-knockout F9 cells, indicating that mammalian Gaa1p is not required for biosynthesis of GPI but essential for attachment of GPI proteins.

When anchors are not attached to proteins that are normally GPI anchored, as is the case in *gaa1* mutant, these proteins are defective for transport to the Golgi apparatus (Nuoffer *et al*., 1991; Moran and Caras, 1992; Micanovic *et al*., 1990). This could be the reason for the indirect effect of the *gaa1* mutation on endocytosis. We can imagine that a GPI anchored protein is required, either directly or because of its enzymatic activity, for endocytosis. In this case, when GPI anchoring is completely blocked, this critical protein would be depleted from its normal location, and a delayed endocytic defect would ensue.

I.7.2.3. Gaa1p and Gpi8p form a protein complex

To test whether the two components of the GPI transamidase form a complex, Gaa1p and a control ER membrane protein ALDH (Masaki *et al*., 1994) were tagged with the FLAG epitope, and Gpi8p and ALDH with GST. The tagged proteins were expressed in various combinations in CHO cells and FLAG-tagged proteins were immunoprecipitated with anti FLAG beads from detergent extracts of the cells. The precipitates were analysed by Western blotting with anti-GST and anti-FLAG to assess coprecipitation. GST-Gpi8p coprecipitated with FLAG-Gaa1p whereas GST-ALDH was not coprecipitated with FLAG-Gaa1p, indicating a specific interaction between Gpi8p and Gaa1p. (Ohishi *et al*., 2000).

The nonfunctional mutant of human Gpi8p lacking the transmembrane domain forms a complex with Gaa1p as shown by a coprecipitation assay, indicating that the transmembrane domain and amino acids 311-321 are not necessary for association with Gaa1p (Ohishi *et al*., 2000).

I.7.2.4. Putative function of Gaa1p

It is not possible to predict functions of Gaa1p from its sequence because it has no significant homology to other proteins of known functions.

In the absence of Gaa1p or Gpi8p, a carbonyl intermediate between the precursor protein and the GPI transamidase was not formed (Ohishi *et al*., 2000; Yu *et al*., 1997). Two steps are involved in generation of the carbonyl intermediate. First, the transamidase recognizes a GPI attachment signal peptide located at the carboxy terminus of the precursor protein and presents it to the catalytic site. Second, the signal peptide should be cleaved by the catalytic site, resulting in formation of a carbonyl intermediate (Maxwell *et al*., 1995). Gpi8p, according to its sequence homology to cysteine proteases, should function in the second step. Gaa1p could recognize the GPI attachment signal peptide. This possibility is supported by the fact that overexpression of yeast Gaa1p could partially suppress the processing defect seen in the GPI signal peptide mutants of Gas1p (Hamburger *et al*., 1995). This hypothesis is also supported by the several hydrophobic regions observed in both yeast and mammalian Gaa1ps which could interact with the required hydrophobic C-terminal domain of the GPI attachment signal.

I.8. Intracellular transport and sorting of GPI-anchored proteins

The mecanisms regulating the sorting processes that operate to target newly synthesized proteins and lipids from their site of synthesis, the ER, to their correct final destinations are only partially understood (Rothman and Wieland, 1996; Schekman and Orci, 1996). Many proteins transported along the secretory pathway contain specific sorting signals. These signals can be recognized by sorting receptors and can be used for the forward transport of the proteins (mannose 6-phosphate receptor (Kornfeld *et al*., 1992)) or for their retrieval to the compartment from wich originate (KDEL receptor (Pelham *et al*., 1995)). In addition, interaction of proteins with lipids can provide a different type of sorting mechanism.

I.8.1. Sorting of GPI-anchored proteins based on their association with the sphingolipid microdomains.

Sphingolipids have been propopsed to be in a liquid-ordered (Lo) phase, since they have a high melting temperature. In contrast, glycerolipids, with a lower melting temperature, seem to be in a liquid-disordered (Ld) phase. In cellular membranes, this difference could

produce a lateral association of sphingolipids, creating a Lo phase, microdomain or raft, surrounded by glycerolipids disposed in a more fluid Ld phase. This idea is consistent with the fact that sphingolipids are found in cold detergent-insoluble extracts (Brown and London, 1998). In addition, a non-detergent-based technique, fluorescence quenching, shows that sphingolipid Lo phase formation occurs in artificial membranes and that this formation correlates well with detergent insolubility (Ahmed *et al*., 1997). Since GPI-anchors contain a saturated acyl chain, GPI-anchored proteins would tend to be associated with sphingolipids rather than glycerolipids. The following data supports this idea: (i) GPI-anchored proteins are found together with sphingolipids and cholesterol in cold detergent-insoluble extracts from cells and from liposomes (Brown and Rose, 1992; Schroeder *et al*., 1994). (ii) Saturated acyl chains in the sphingolipids are required for the presence of GPI-anchored proteins in cold detergent-resistant extracts (Schroeder *et al*., 1994). (iii) The insolubility of GPI-anchored proteins depends on the abundance of sphingolipids and cholesterol in artificial lipid mixtures.

In mammalian cells, it has been observed that when the synthesis of ceramide (the precursor of the sphingolipids) is decreased by incubation with fumonisin (Wang *et al*., 1991), GPI-anchored proteins are specifically mis-sorted in MDCK cells and in primary hippocampal neurons (Mays *et al*., 1995; Ledesman *et al*., 1998). In addition, cholesterol is required for the selective transport of a GPI-anchored protein to the cell surface (Hannan and Edidin, 1996). These experiments are in agreement with original model which suggests that, in mammals, sphingolipid microdomains recruit GPI-anchored proteins at the *trans*-Golgi network (TGN) and somehow determine which type of transport vesicle they enter: the ones going to the basolateral or the ones going to the apical plasma membrane (Simons and van Meer, 1988).

In yeast, ceramide is required for specific transport of GPI-anchored proteins from the ER to the Golgi (Horvarth *et al*., 1994; Skrzypek *et al*., 1997; Sütterlin *et al*., 1997). When yeast cells are incubated with myriocin, an inhibitor of ceramide synthesis, or when *lcb1-100* (serine palmitoyl-transferase, the first enzyme in ceramide biosynthesis) mutant cells are incubated at non-permissive temperature, the ER to Golgi transport of the GPI-anchored protein Gas1p becomes defective. This ceramide requirement is specific for GPI-anchored proteins because the transport of non-GPI anchored proteins remains normal under these conditions. From these results it has been proposed that, in yeast, GPI-

anchored proteins may be sorted from non-GPI anchored proteins in the ER by selective recruitment in ceramide-rich microdomains.

This leads to an apparent discrepancy concerning the point in the secretory pathway where GPI-anchored proteins are incorporated into the ceramide/sphingolipid domains. In the mammalian model, the initial sorting occurs in the Golgi and, for the yeast model, sorting occurs in the ER.

One clear difference between mammals and yeast is the length of the acyl chains of sphingolipids (Dickson, 1998) and GPI-anchors (McConville and Ferguson, 1993). In yeast, ceramide contains a very long saturated acyl chain (C26) which may favor the clustering of ceramides and the formation of thicker microdomains in the ER. In yeast, the lipid moiety of the GPI anchor is exchanged from diacylglycerol to ceramide (C26) or to a more hydrophobic diacylglycerol with a very long chain fatty acid (C26). These remodeling events can occur in the ER (Sipos *et al*., 1997). Thus, the long acyl chains could act to sort the GPI-anchored proteins into the thicker ER ceramide microdomains and, consequently, drive the incorporation of these proteins into distinct ER-derived vesicles (Reggiori *et al*., 1997). Consistent with this hypothesis, *elo2* and *elo3* (fatty acid chain elongation machinery in yeast) mutants are defective specifically in transport of GPI-anchored proteins from the ER to the Golgi (David *et al*., 1998).
In mammals, the acyl chain of ceramides (C18-C24) and GPI-anchors are shorter than in yeast. Thus, the thickness-based sorting mechanism would not be predicted to take place in the ER. However, it could still occur in the Golgi (Munro, 1998), where the domains of the membrane containing sphingolipids can be thicker than in the ER, because cholesterol is more abundant there and sphingolipid is able to form thicker bilayers in the presence of cholesterol (Nezil and Bloom, 1992; Maulik and Shipley, 1996).
Another difference between yeast and mammals is that the hydroxylation of the fatty acid of ceramide is common in yeast but rare in mammals (Dickson, 1998). These polar groups could be utilized to form hydrogen bonds between adjacent ceramides or ceramides and GPI-anchors. This could increase the efficiency of ER clustering of ceramides in yeast but not in mammals. Mammalian ceramides would then cluster in the Golgi where they are converted to sphingolipids, whose head groups are involved in hydrogen bonds.

I.8.2. Sorting of GPI-anchored proteins based on GPI anchor

In addition to the lipid-mediated sorting, transport of GPI-anchored proteins from the ER to the plasma membrane presents other specific characteristics. GPI anchoring has been proposed to be a requirement for exit from the ER. In yeast, GPI-anchored protein transport from the ER to the Golgi compartment shows at least two specific requirements (Sütterlin *et al*., 1997). First, *ret1-1* (hereafter refered to as *cop1-1*), a mutant in the α subunit of coatomer (COPI), specifically blocks the transport of GPI-anchored proteins without affecting transport of other secretory proteins. This block is not due to a defect in GPI anchoring or remodeling. Second, ongoing synthesis of sphingoid bases and/or ceramide plays a critical role in the ER to Golgi transport of Gas1p and Yps1 (previously referred to as Yap3p), which are GPI-anchored proteins. When yeast cells are incubated with myriocin (Horvarth *et al*., 1994), an inhibitor of ceramide synthesis, or when *lcb1-100* cells (conditional mutant in serine palmitoyltransferase activity, the first enzyme in ceramide biosynthesis (Zanolari *et al*., 2000)) are incubated at nonpermissive temperature (Sütterlin *et al*., 1997), the ER to Golgi transport of two GPI-anchored proteins, Gas1p and Yps1p, is defective. Other secretory proteins, such as gpαF, invertase, or CPY are not affected. Another experiment shows that when GPI anchoring was not completed in yeast microsomes, the unanchored proteins were not included into newly formed, ER-derived vesicles (Doering and Schekman, 1996). It has been assumed for many years that secretory protein sorting occurs only after arrival to or exit from the Golgi compartment. Recently an in vitro assay that reconstitutes a single round of budding from the ER shows that GPI-anchored proteins and other secretory proteins exit the ER in distinct vesicles (Muniz *et al*., 2001).

I.8.3. Receptor-mediated sorting of GPI-anchored proteins

Several lines of evidence suggest that cargo proteins are incorporated selectively in the ER into COPII-coated vesicles to be transported to the Golgi (Mizuno and Singer, 1993; Balch *et al*., 1994; Kirchhausen *et al*., 1997; Herrmann *et al*., 1999). This sorting process is driven by the interactions between the secretory proteins and the COPII components (Aridor *et al*., 1998; Kuehn *et al*., 1998). GPI-anchored proteins are lumenal. Therefore, they are not able to interact directly with the COPII coat on the cytosolic face of the

budding membrane. In this case, the COPII-dependent sorting model implies the existence of a transmembrane adaptor(s) to connect the lumenal GPI-anchored proteins with the cytosolic coat components. Emp24 and Erv25p, members of the p24 family in yeast, are good candidates to function as adaptors/receptors for the GPI-anchored proteins. Both proteins form part of the same complex, they are major components of the COPII-coated vesicles and they are required for the efficient transport of Gas1p from the ER to the Golgi (Schimmoller *et al*., 1995; Belden and Barlowe, 1996). These putative receptors may not be exclusive for GPI-anchored proteins, because the ER to Golgi transport of the soluble protein, invertase, is reduced *in vivo* in *emp24* and *erv25* mutants. In yeast, the transport from the ER to the Golgi of GPI-anchored proteins is inhibited strongly in mutants of α-COP (ret1-1), a component of the COPI coat (Sütterlin *et al*., 1997). COPI has been shown to be involved in retrograde transport from the Golgi to the ER (Letourneur *et al*., 1994). Thus it is conceivable that coatomer could function in the retrival of some specific factor(s) from the Golgi to the ER required for the forward transport of GPI-anchored proteins (Sütterlin *et al*., 1997).

I.8.4. The ω-minus region mediated sorting of GPI-anchored proteins

Two kinds of amino acid sequences in the region composed of five amino acids upstream of the ω site (the ω-minus 5-1 region) have been proposed to be responsible for the final destination of the GPI-anchored proteins in *S.cerevisiae*: dibasic residues for remaining on the plasma membrane (Caro *et al*., 1997; Vossen *et al*., 1997) and specific amino acid residues at sites 4 or 5 and 2 upstream of the ω site (ω-4/5 and ω-2 sites) for incorporation into the cell wall (Hamada *et al*., 1998).

All yeast GPI anchored proteins that are known not to be covalently linked to the cell wall, including Gas1p, contain a dibasic amino acid motif (R and/or K), which is located just N terminal from their ω site (Table 4).

Hamada *et al*. (1998a) showed some sequence similarity in the ω-minus region of 15 GPI-dependent cell wall proteins where either Val or Ile occupies the ω-5 site and Tyr is predominant at the ω-2 site (see table 4). S.*cerevisiae* Yap3p, an aspartic protease, is a GPI-anchored plasma membrane protein (Ash *et al*., 1995) which was used by Hamada *et al*. (1998b) to test wheter the replacement of amino acid residues at the ω-4 or -5 and ω-2 sites by the conserved amino acids of cell wall proteins directs the protein into the cell

wall. A fusion protein with the C-terminal sequence of Yap3p was constructed and was not detected in the cell wall as a glucanase-extractable protein; changes of the ω-minus region from the wild type TSSKR to VSSVS, SVSVS, ISSYS, or VSSYS resulted in the fusion protein being incorporated into the cell wall. Taken together, these results showed that sequences containing Val or Ile at the ω-4 or -5 site and Val or Tyr at the ω-2 site are determinants for the cellular localization of the GPI-associated proteins; this sorting point is called the ω-4/5 and ω-2 rule.

Table 4. Analysis of cell wall and plasma membrane-associated proteins

YDR534C	IFTNGKSSTTPQ**I**VN**Y**T	G	AADSIAAGTGLMGAALAAVIFL*
YIR019C/FLO11	SGSAVATYSVPSISST**Y**Q	G	AANIKVLGNFMWLLLALPVVF*
YNL327W/ETG2	ITWYSSSTIKPPS**I**ST**Y**S	G	AAGQLTIRIGSLLLGLISFLL*
YOR214C	PTITPGNITT**I**GG**Y**E	N	SSSSLMPSMGILSFLFGLYLLLHP*
YDR134C	TEKPTQQGSSTQT**V**T**S**Y**T**	G	AAVKALPAAGALLAGAAALLL*
YPL130W	TPYSNISSLNED**Y**D	N	ASNFLTPTTVALAVLLTILLFIQAY*
YOR009W	TVVSVQSKTTG**I**V**E**QTE	N	GAAKAVIGMGAGALAAVAAMLL*
YER150W	KPTSETSVSSTHD**V**ET**N**S	N	AANARAIPGALGLAGAVMMLL*
YDR077W/SED1	STVVPVSSSASSHS**V**VI**N**S	N	GANVVVPGALGLAGVAMLFL*
YOR383C	TSSSATSSSTAE**L**SS**Y**T	G	AADAITAGTGLMGAALAAVMLL*
YJR151C	SIAHSSASYT**V**SI**N**T	N	GAYNFDKDNIFGTAIVAVVALLLL*
YJR004C/Agα1	IQQNFTSTSLM**I**ST**Y**E	G	KASIFFSAELGSIIFLLLSYLLF*
YJL078C/PRY3	TSTTAK**L**SA**Y**E	G	AATPLSIFQCNSLAGTIAAFVVAVLFAF*
YLR110C	APKNTTSAAPTHS**V**T**S**Y**T**	G	AAAKALPAAGALLAGAAALLL*
YNL300W	SISSQANTTTHE**I**ST**Y**V	G	AAVKGSVAGMGAIMGAAAFALL*

YOL030W/GAS5	SLLKSAASATSSSQSSS***K*S*K**	G	AAGIIEIPLIFRALAELYNLVL*
YDR055W	SSGASSSSS***K*S*K***G	N	AAIMAPIGQTTPLVGLLTAIIMSIM*
YBR078w/ECM33	AQANVSASASSSSSSS***K*K*S*K**	G	AAPELVPATSFMGVVAAVGVALL*
YNL190W	GPGE***K*A*R*K**N	N	AAPGPSNFNSIKLFGVTAGSAAVAGALLLL*
YDR144C/YAP2	SSTGMLSPTSSSST***R*K**E	N	GGHNLNPPFFARFITAIFHHI*
YIR039C/YAP6	SSFSSSGGSSESTT***K*K**Q	N	AGYKYRSSFSFSLLSFISYFLL*
YLR194C	YKSTVNGKVASVMSNST	N	GATAGTHIAYGAGAFAVGALLL*
YLR120C/YAP3	SGNLTTSTASATSTSS***K*R**	N	VGDHIVPSLPLTLISLLFAFI*
YDR522C/SPS2	GKNGAKSQGSS***K*K**ME	N	SAPKNIFIDAFKMSVYAVFTVLFSIIF*
YMR215W/GAS3	TGSSSASSSS***K*S*K**	G	VGNIVNVSFSQSGYLALFAGLISALL*
YMR008C/PLB1	ASGSSTH***K*K**	N	AGNALVNYSNLNTNTFIGVLSVISAVFGLI*
YOL132W/GAS4	EDADEDKDDL***K*R*K**H***R**	N	SASISGPLLPLGLCLLFFTFSLFF*

The potential GPI attachment site (ω-site) of each sequence is indicated in the middle, sandwiched between spacer regions. The amino acids residues I, V, and L at the ω-5 site and Y and N at the ω-2 site are marked in bold (cell wall-associated proteins, top), and the basic amino acid residues K and R in the short ω minus region (ω-5 to ω-1) are marked in bold plus italic (plasma membrane-associated proteins, bottom). The potential N-glycosylation sites are double underlined. Derived from (Hamada *et al*., 1999).

I.8.5. Cell wall incorporation of GPI-anchored proteins

The cell wall of *S.cerevisiae* is made up of three components: glucan, mannoproteins, and chitin (Klis *et al*., 1994). The former two are the major components of the cell wall. The mannoproteins can be divided into three groups: the sodium dodecyl sulfate (SDS)-extractable mannoproteins, glucanase-extractable mannoproteins, and reducing agent-extractable mannoproteins. The glucanase-extractable mannoproteins are covalently bound to β-1,6 glucan and are released only by digestion of glucan with glucanase (De Nobel and Lipke, 1994). They have three characteristics in common: a N-terminal signal sequence for secretion, a serine/threonine-rich sequence which provides sites for glycosylation, and a C-terminal GPI-attachment signal. Whereas GPI-proteins linked to the plasma membrane possess an intact GPI-anchor, GPI proteins in the cell wall have their GPI-anchor trimmed at the plasma membrane, prior to incorporation into the cell wall (Lu *et al*., 1994,1995; Müller *et al*., 1996). The exact structure of the GPI-remnant present in mature cell wall proteins is still unknown, but it lacks at least the phospholipid part. Therefore, in the case of cell wall proteins, the phospholipid of the GPI-moiety is not the anchoring structure. Instead, the glycan part of the GPI-remnant has been shown to be bound to the cell wall glucans (Kapteyn *et al*., 1997) (Figure 23).

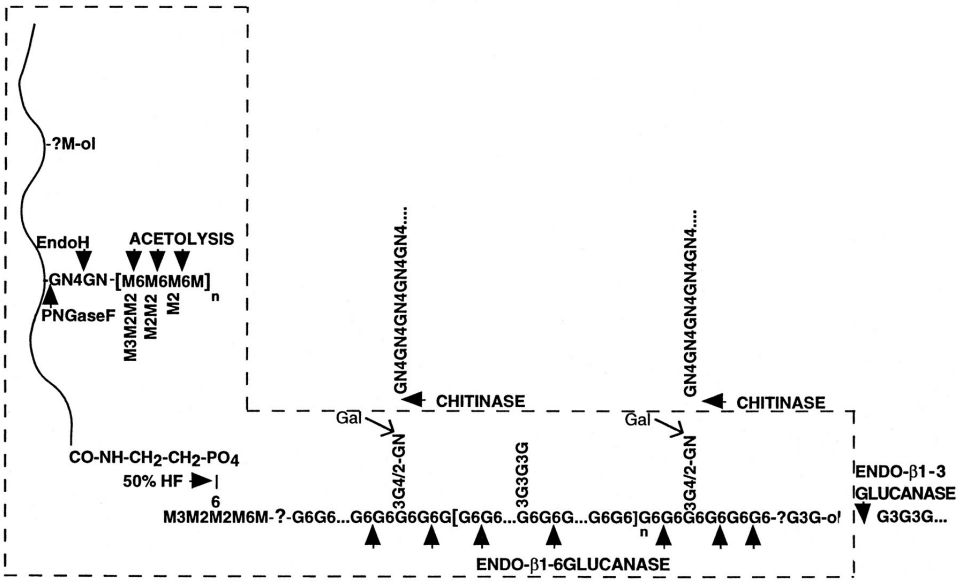

Fig.23. Cell wall incorporation of GPI-anchored proteins . Chains of chitin and β-1,3 glucan appear outside the broken line. Protein is drawn as a curved line, and the glycan

part of the GPI-remnant (M3M2M2M6M) appears on the left. Linkages between sugars are indicated with a single number, e.g. G6G stands for glucose1-6glucose. The positions at which different enzymes cut the chains are shown with arrowheads. G, glucose; M, mannose; GN, N-acetylglucosamine. (Kollar *et al*., 1997).

I.9. Extracellular transport and recycling of GPI-anchored proteins.

I.9.1. Phospholipase mediated secretion of GPI-anchored proteins

Brunner *et al*. (1991) showed that in human bone marrow stromal cells, the GPI-anchored heparan sulfate proteoglycan is cleaved and released in the extracellular medium by a phospholipase D, this action representing an important event in regulating hematopoiesis. The GPI-anchored complement regulatory protein, called decay accelerating factor (DAF), was shown to be cleaved and released by a GPI-PLD in HeLa cells (Metz *et al* ., 1994); the soluble DAF can then inhibit fluid phase activation of the complement cascade.
Another GPI-anchored membrane protein, the 120 kDa form of Neural cell adhesion molecule (NCAM) was shown to be cleaved and released by a phospholipase (He *et al*., 1987). NCAM-120 can mediate axon outgrowth, cell pattern formation, and nerve-muscle interaction, and the changing amounts of soluble form of NCAM-120 found in the brain during development suggest an important role of this protein in this development process. Carcinoembryonic antigen (CEA), the receptor for ciliary neurotrophic factor (CNTFRα), and Tag-1, a growth and guidance factor of axons, are other GPI-anchored proteins cleaved and released by a phospholipase C or D (Benchimol *et al*., 1989; Davis *et al*., 1993; Furley *et al*., 1990).

I.9.2. Proteolytic cleavage-mediated secretion of GPI-anchored proteins

Alkaline phosphatase, a GPI-anchored protein, was shown to be released by a protease from BC3H1 myocytes, after stimulation of the cells with insulin (Romero *et al*., 1988). The conclusion that a protease is responsible for release is based on the observation that the protease inhibitor, p-aminobenzoic acid blocks the effect of insulin.

The GPI-anchored folate receptor, an essential component of normal folate uptake and metabolism, was shown to be constitutively converted by a metalloprotease to a soluble molecule in a variety of tissues (Elwood *et al.,* 1991). The soluble folate receptor may play a role in folate transport and hepatic uptake.
CD14 is a GPI-anchored receptor for the serum lipopolysaccharide, expressed on the surface of monocytes/macrophages, and shown to be cleaved and released by a protease (Bazil *et al*., 1991).

I.9.3. Endocytosis

GPI-anchored proteins are internalized via a non-clathrin-mediated endocytic route (Sabharanjak and Mayor, 1999) and are delivered to peripheral tubular-vesicular endosomes called GEECs (GPI-anchored proteins enriched endosomal compartments). These endosomal organelles, which contain a significant fraction of internalized fluid phase, deliver GPI-anchored proteins to the pericentriolar recycling endosomal compartment (REC). GPI-anchored proteins do not seem to be delivered to late endosomes, consistent with observations made in several studies (Keller *et al*., 1992; Mayor *et al.,* 1998). After GPI proteins have reached the REC, the recycling to the cell surface does not seem to be mediated by any specific signal but was shown to be three to four times slower than that of other components of the recycled membrane (Mayor *et al*., 1998). This retention in the REC in preference to other recycling membrane proteins requires the presence of a GPI anchor and is cholesterol and sphingolipid dependent, implicating a raft-based mechanism for segregation of GPI-anchored proteins in the REC (Chatterjee *et al*., 2001). GPI-anchored proteins, in ER to Golgi traffic (Muniz *et al*., 2000), exit the REC via distinct vesicular or tubular carriers (Chatterjee *et al*., 2001). The ability of the GPI anchor to retain proteins in this compartment reveals an important function of this post-translational modification. Essential functions of many GPI-anchored proteins are likely to be mediated by the endocytic retention. For exemple, folate uptake via the human folate receptor (FR-GPI) is severely impaired in cholesterol or sphingolipids-depleted cells in which FR-GPI is no longer retained in endosomes (Chatterjee *et al*., 2001). Other GPI-anchored proteins like prions, were shown to be endocytosed. Prions are the infectious component of scrapie, a degenerative neurological disease of sheep and mice, similar to the human neurological disorders, Kuru, Creutzfeld-Jacob, and Gerstmann-Sträussler syndrome. The non infectious form of prion is expressed

at the cell surface whereas the infectious form of prion accumulates within an intracellular compartment that appears to be related to the Golgi apparatus (Borchelt *et al*., 1990).

I.9.4. Intercellular transfer of GPI-anchored proteins between cells

Rifkin and Landsberger (1990) observed that the variant surface glycoprotein (VSG) of the African trypanosome diffused onto the surface of erythrocytes, sensitizising the erythrocytes to lysis by complement, a phenomenon accounting in part for the anemia observed in African trypanosomiasis.
Another parasite, *Schistosoma mansoni*, acquires the GPI-anchored complement regulatory factor (DAF), thus protecting himself from destruction by complement (Fatima *et al*., 1991). Another example comes from sperm maturation in which some of the GPI-anchored proteins are not synthesized by the spermatozoa themselves, but by cells of the male genital tract; they are transported to the fluid secretions, possibly associated with membrane vesicles, and then incorporate themselves into the sperm membrane (Kirchhoff and Hale, 1996).

I.10. Biological functions of GPI-anchored proteins and free GPIs

In men, GPI anchored proteins are involved in cell to cell adhesion and development (Minchiotti *et al*., 2000), activation and control of inflammation and immune responses (Sendo *et al*., 1999; Okazaki and Moss, 1998), reproduction (Kirchhoff *et al*., 1996) , signal transduction (Kramer *et al*., 1999), as well as in iron transport (Fortna *et al*., 1999).

In yeast, GPI anchored proteins can be classified in several functional categories : agglutinin like proteins, flocculation promoting proteins, aspartyl protease like proteins, cell wall biosynthesis promoting proteins (Mouyna *et al*., 2000), and SRP family-like proteins (Hamada *et al*., 1998).
α-agglutinins and a-agglutinins are complementary cell adhesion molecules that mediate cell to cell interaction during yeast mating (Lipke and Kurjan, 1992). Aspartyl proteases

are represented by Yap3p, a GPI anchored protein for which proteolytic activity has been demonstrated in vitro and in vivo; the physiological substrates have not been identified (Cawley *et al*., 1993; Bourbonnais *et al*., 1993). Biochemical assays performed on purified recombinant Gas1p, showed that this protein has a 1,3-beta-glucanosyltransferase activity similar to that of Gel1p, a novel 1,3-beta-glucanosyltransferase isolated from the cell wall of Aspergillus fumigatus. This enzyme is an endo1,3-beta-glucanase and transfers the newly generated reducing end to the non-reducing end of another 1,3-beta-glucan molecule forming a 1,3-beta linkage, resulting in the elongation of 1,3-beta-glucan chains. Thus Gas1p plays an active role in fungal cell wall synthesis (Mouyna *et al*., 2000).

I.11. GPI-anchored proteins and human diseases.

I.11.1. Alzheimer disease

The amyloid that is invariably deposited in Alzheimer's disease is composed of an approximately 4-kDa peptide (amyloid β-peptide, Aβ) that is derived from a larger protein, the amyloid β protein precursor, by cleavage of its N- and C-terminal ends by unidentified β- and γ- secretase, respectively. In yeast, αsecretase activity has been attributed to GPI-anchored aspartyl proteases. Sambamurti *et al*. (1999) demonstrated, after treatment of several cell lines by PI-PLC, that one or more GPI-anchored proteins play an important role in β-secretase activity and Aβ secretion in mammalian cells. They concluded that the cell surface GPI-anchored proteins involved in Aβ biogenesis may be excellent therapeutic targets in Alzheimer's disease.

I.11.2. Prion diseases

Prion diseases are a group of neurodegenerative diseases that include Creutzfeld-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia and Kuru in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle. These diseases are caused by a conformational change in the normal cellular isoform of the prion (PrP^C)

to the scrapie isoform (PrP^{Sc}) (Prusiner, 1998). PrP^{C} contains two N-glycosylation sites and a GPI anchor attachment site. It was shown that the GPI anchor is required for N-glycosylation to occur, leading to the conclusion that glycosylation and membrane anchorage are co-operative processes. It was also shown that under-glycosylated molecules appear to be preferred substrates in the generation of PrP^{Sc}, revealing an important indirect role of the GPI-anchor in the folding, stability and transport of PrP^{C} (Walmsley *et al*., 2001)

I.11.3. African sleeping sickness

African sleeping sickness is a debilitating and often fatal disease caused by African trypanosomes transmitted by tsetse fly. These extracellular protozoan parasites survive in the human bloodstream by virtue of a dense cell surface coat made of variant surface glycoprotein. The parasites have a repertoire of several hundred immunologically distinct GPI-anchored variant surface glycoproteins and they evade the host immune response by antigenic variation. The development of vaccines is limited by this antigenic variation, so that compounds that inhibit the assembly or transfer of the GPI anchors are potential trypanocidal products (Ferguson *et al*., 1999).

I.11.4. Malaria

Malaria, a life-threatening disease caused by parasitic protozoa of the genus *Plasmodium*, is a major health problem throughout the tropical and subtropical regions of the world. Among the four species that infect humans, *Plasmodium falciparum* is the most virulent. A study describing the characterization of the carbohydrates in intraerythrocytic *P. falciparum* proteins established that GPI anchors represent the major carbohydrate modification of cell surface proteins (Gowda *et al.,* 1997). This post-translational modification plays an important role in antigenicity and is an important report for developing vaccines based on antigens of the blood stage parasit. Moreover, components of the malarial GPI anchor are released and stimulate the nonspecific immune response, an effect which guarantees that the infected host survives and thus, becomes a permanent habitat for the parasite.

I.11.5. Paroxysmal Nocturnal Hemoglobinuria disease

Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired clonal disorder in which intravascular hemolysis results from the somatic mutation of the totipotent stem cells causing an intrinsic defect in the red cell membrane. PNH cells lack GPI-anchored membrane proteins, caused by a somatic mutation of the X-linked PIG-A gene which participate in an early step of GPI anchor synthesis. The hemolysis is due to the lack of DAF and CD59, two GPI anchored proteins which normally are required for the inactivation of complement components. PNH is also characterized by recurrent life threatening venous thromboses, an intimate association with aplastic anemia and bone marrow failure (Chrobak, 2000).

I.11.6. Chronic gastritis caused by *Helicobacter pylori*

Helicobacter pylori is a microaerophilic, spiral-shaped, Gram negative bacterium that colonizes the gastric mucosa of 50-60% of the world's population. *H.pylori* secrete a vacuolating toxin (VacA) which plays a major role in the development of chronic gastritis, peptic ulcer, and gastric cancer. VacA binds to specific, high affinity cell surface receptors, is internalized by cells via a temperature dependent process, and localizes in the endocytic-endosomal compartment from which vacuoles originate. It has been reported that VacA may act as a channel-forming toxin and that VacA channels play a direct role in cell vacuolation. The high sensitivity of Hep-2 cells to VacA is abolished by treating the cells with PI-PLC, suggesting that one or several GPI-anchored proteins are involved in the pathogenycity (Ricci *et al*., 2000).

I.11.7. *Aeromonas hydrophila* toxin

Aeromonas hydrophila, a member of the *Vibrionaceae* family, is a bacteria which secretes a pore forming toxin, aerolysin. The bacterium causes a variety of infections ranging from gastroenteritis to deep wound infection and septicemia. The toxin is secreted as a dimeric inactive precursor (proaerolysin), activated by host cell proteases which remove a C-terminal peptide, and then binds to the surface of the erythrocyte via a 47-kDa

GPI-anchored glycoprotein that act as a receptor. The toxin causes dramatic vacuolation of the ER, but does not affect other intracellular compartments (Abrami *et al*., 1998).

I.12. Introduction to the thesis work

The principal aim of the thesis was the functional analysis of the yeast *Saccharomyces cerevisiae* Gpi8 protein and the characterization of the purified GPI-transamidase complex. Gpi8p has been shown to be essential for the GPI anchor addition onto newly synthesized proteins in the ER (Benghezal *et al*., 1996). Gpi8p has also been shown to show significant homology to a family of vacuolar plant endopeptidase, one of which is supposed to catalyse a transamidation step in the maturation of ConcavalinA. For thus, Gpi8p has been assimilated to a transamidase which catalyzes the transfer of GPI anchors onto proteins. The first step in the thesis has been the development of an *in vitro* test to give an evidence that Gpi8p has a proteolytic activity. For this, Gpi8p has been purified to purity in *E.coli* and tested against peptide substrates reproducing the GPI anchoring site. Several truncated forms of Gpi8p has also been constructed to determine essential domains for the activity of the enzyme. Gaa1p has also been shown to be essential for the transfer of GPI anchors onto proteins (Hamburger *et al*., 1995), and was suspected to interact directly with Gpi8p. We developed a GAL4-two-hybrid system to study the interaction between both proteins when expressed in physiological levels. The second step of the thesis was the characterization of the transamidase complex. For this, the complex was purified and each component identified by mass spectrometry. Finally, we propose a two-hybrid-based model for the macromolecular organization of the transamidase complex. The collection of these data and the characterization of the GPI transamidase complex has been planified in view of future studies to better understand the enzymatic machinery of GPI anchor post-translational modification of proteins by yeast cells.

II. RESULTS

II.1. Identification of Gpi8p-interacting partners in the GPI Transamidase Complex

Article:

The GPI Transamidase Complex of Saccharomyces cerevisiae Contains Gaa1p, Gpi8p and Gpi16p

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The GPI Transamidase Complex of *Saccharomyces cerevisiae* Contains Gaa1p, Gpi8p, and Gpi16p

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Gpi8p and Gaa1p are essential components of the GPI transamidase that adds glycosylphosphatidylinositols (GPIs) to newly synthesized proteins. After solubilization in 1.5% digitonin and separation by blue native PAGE, Gpi8p is found in 430–650-kDa protein complexes. These complexes can be affinity purified and are shown to consist of Gaa1p, Gpi8p, and Gpi16p (YHR188c). Gpi16p is an essential N-glycosylated transmembrane glycoprotein. Its bulk resides on the lumenal side of the ER, and it has a single C-terminal transmembrane domain and a small C-terminal, cytosolic extension with an ER retrieval motif. Depletion of Gpi16p results in the accumulation of the complete GPI lipid CP2 and of unprocessed GPI precursor proteins. Gpi8p and Gpi16p are unstable if either of them is removed by depletion. Similarly, when Gpi8p is overexpressed, it largely remains outside the 430–650-kDa transamidase complex and is unstable. Overexpression of Gpi8p cannot compensate for the lack of Gpi16p. Homologues of Gpi16p are found in all eucaryotes. The transamidase complex is not associated with the Sec61p complex and oligosaccharyltransferase complex required for ER insertion and N-glycosylation of GPI proteins, respectively. When GPI precursor proteins or GPI lipids are depleted, the transamidase complex remains intact.

INTRODUCTION

Many glycoproteins of lower and higher eucaryotes are attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI) (McConville and Ferguson, 1993; Kinoshita and Inoue, 2000). GPI anchoring of proteins is essential for the growth of *Saccharomyces cerevisiae* (Leidich *et al.*, 1994; Schönbächler *et al.*, 1995). *S. cerevisiae* contains ~70 open reading frames predicting GPI proteins, and many of these have been found to be cell wall glycoproteins (Caro *et al.*, 1997; Hamada *et al.*, 1998).

Precursors of GPI-anchored proteins have a classic signal sequence for import into the ER at their N-terminus and a GPI-anchoring signal at their C-terminus; the C-terminal signal is necessary and sufficient to direct GPI addition (Caras *et al.*, 1987). The C-terminal GPI-anchoring signal is recognized and removed by a GPI transamidase, which replaces it by a preformed GPI. GPI-anchoring signals are composed of a C-terminal hydrophobic domain, which is separated by a short hydrophilic spacer from the cleavage/attachment site (ω site) (Moran and Caras, 1991; Gerber *et al.*, 1992; Nuoffer *et al.*, 1993).

The transfer of preformed GPIs onto proteins has been studied in microsomal translation/translocation/GPI-anchoring systems (Ramalingam *et al.*, 1996; Sharma *et al.*, 1999) or translocation/GPI-anchoring systems (Doering and Schekman, 1997) in several organisms, and these studies allowed a preliminary biochemical characterization of the GPI transfer reaction. The GPI transferase is believed to act as a transamidase, i.e., to jointly remove the GPI-anchoring signal and transfer the preformed GPI (Ramalingam *et al.*, 1996; Sharma *et al.*, 1999). Genetic approaches have identified genes required for the addition of GPI anchors. Transamidase-deficient cells are expected to accumulate complete GPIs as well as GPI precursor proteins retaining the GPI-anchoring signal. This phenotype is exhibited by two yeast mutants, *gaa1* and *gpi8* (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996). *GAA1* is essential and encodes a 70-kDa ER protein with a large, hydrophilic, lumenal domain, followed by several transmembrane domains (TMDs) and a cytosolic ER retrieval signal on its extreme C-terminus. *GPI8* is also an essential gene and encodes a type I ER membrane protein with a single TMD. Gpi8p has 25–28% homology to a family

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Abbreviations used: GPI, glycosylphosphatidylinositol; GST, glutathione-s-transferase; TMD, transmembrane domain; wt, wild-type.

of cysteine proteinases, one of which is able to act as a transamidase (Benghezal *et al.*, 1996). The Cys and His residues predicted to be active sites by sequence comparison with caspases indeed are essential, and their mutation to Ala yields nonfunctional *GPI8* alleles (Chen *et al.*, 1998; Meyer *et al.*, 2000; Ohishi *et al.*, 2000). Recent evidence shows that Gpi8p may be part of a larger protein complex. First, the human Gpi8p is efficiently coimmunoprecipitated with human Gaa1p when tagged forms are coexpressed in CHO cells, and even truncated versions of Gpi8p lacking the C-terminal TMD still can be coprecipitated with Gaa1p (Ohishi *et al.*, 2000). Second, the overexpression in wild-type (wt) cells of any *GPI8* allele that is mutated in one of the active site residues leads to cell growth arrest and causes the accumulation of unprocessed GPI lipids and protein precursors (Meyer *et al.*, 2000). Here we isolate the complex and analyze its components.

MATERIALS AND METHODS

Strains, Growth Conditions, and Materials

Yeast strains were *S. cerevisiae* W303–1B (*MAT***α** *ade2–1 can1–100 ura3–1 leu2–3,112 trp1–1 his3–11,15*), FBY525 (*MAT***a** *ade2–1 ura3–1 leu2–3,112 trp1–1 his3–11,15 lys*$^-$ Δ*gpi8::kanMX* containing plasmid YEp*GPI8*), FBY164 (*MAT***α** *ade2–1 leu2–3,112 his3–11,15 gpi8::kanMX2 ura3–1::URA3-GAL1,10*$_{UAS}$*-GPI8*), RH932 (*MAT***a** *gaa1 leu2 ura3 bar1–1*) (Hamburger *et al.*, 1995), 521–17A-H42 (*MAT***a** *mcd4 trp1–289 leu2 ura3–52*) (Packeiser *et al.*, 1999), CWH4 (*MAT***α** *gpi1 ura3–52 lys2*) (this mutant was isolated as *cwh4* by Frans Klis; it is allelic to *gpi1*; I. Imhof, unpublished data), FBY656 (*MAT***a** *ade2–1 ura3–1 leu2–3,112 trp1–1 his3–11,15 lys*$^-$ *GPI8*Δ*::kanMX2* containing YCplac22-*GST-GPI8*), FBY733b (*MAT***a** *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 YHR188c::kanMX4* containing plasmid pYES2-*GPI16*), FBY735 (*MAT***a** *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 YHR188c::kanMX4* containing plasmid YCplac111-*GAL1*$_{UAS}$*-GPI16*), Y22882 (*MAT***a**/**α***; his3*Δ*1/ his3*Δ*1; leu2*Δ*0/leu2*Δ*0; lys2*Δ*0/LYS2; MET15/met15*Δ*0; ura3*Δ*0/ura3*Δ*0; YHR188c::kanMX4/YHR188c*, derived from BY4743, obtained from EUROSCARF), WADE060−01A(A) (*MAT***a** *ura3–1 his3–11 leu2–3,112 trp1*Δ*2 ade2–1 can1–100 YJR015w(-11, 1503)::kanMX4* in W303 background), and FBY91 (*sec18 gpi8–1 leu2*). Yeast strains were cultured as previously described (Benghezal *et al.*, 1996). SDaa medium is SD medium containing 20−60 mg/l of each of the 20 amino acids, SGaa is the same but with galactose used instead of glucose. Antibodies against Gpi16p were obtained by immunizing rabbits with an octameric peptide corresponding to residues 255–281 of Gpi16p. Antibodies were used after affinity purification on the same peptide. All peptides were synthesized by Alta Bioscience (University of Birmingham, UK). Oligonucleotide synthesis and DNA-sequencing services were provided by Microsynth (Balgach, Switzerland).

Construction of Vectors

Plasmid pYES*-GPI16:** The open reading frame of YHR188c was amplified by PCR with the use of a forward primer that introduces a *Kpn*I and a *Sal*I at the 5′ end (5′-acgttgagctggatcc*ggtaccgtcga-catg**atcctcacactggcctatttcatgctg-3′) and a reverse primer introducing a *Xho*I site at the 3′ end (5′-acgttgagct*ctcgag***tta**gtctgtttttagtctttttttttccctag-3′). The PCR fragment was digested with *Kpn*I/*Xho*I and was inserted into the multiple cloning site (MCS) of the pYES vector (Invitrogen, Carlsbad, CA), which was opened with *Kpn*I/*Xho*I, thus yielding the plasmid pYES-*GPI16*.

YC*plac111-*GAL1*$_{UAS}$-GPI16:** The *GAL1* promoter, i.e., nucleotides −453 to −1 of *GAL1* of *S. cerevisiae* was amplified by PCR with the use of a forward primer that introduces a *Sph*I site at the 5′ end (5′-acgttgagct*gcatgc*acggattagaagccgccgag-3′) and a reverse primer introducing a *Sal*I site at the 3′ end (5′-acgttgagct*gtcgac*ggatccggggttttttctcc-3′). The open reading frame plus the transcription-terminating region of YHR188c was amplified by PCR with the use of a forward primer introducing a *Sal*I site at the 5′ end (5′-acgttgagctggatccggtacc*gtcgacatg**atcctcacactggcctatttcatgctg-3′) and a reverse primer introducing a *Kpn*I site at the 3′ end (5′-acgttgagct*ggtacc*cgatatatttcaattacgacttgttaataaaag-3′). The *GAL1* promoter fragment and the YHR188c fragment were digested with *Sph*I/*Sal*I, and *Sal*I/*Kpn*I, respectively, and both fragments were inserted into the MCS of YCplac111, which was opened with *Sph*I/*Kpn*I, thus yielding YCplac111-*GAL1-GPI16.*

YCplac22-GST-GPI8: harbors GPI8 under the control of the physiological *GPI8* promoter containing the gene for glutathione-s-transferase (GST) inserted behind the N-terminal signal sequence of Gpi8p. For its construction, a *BsiWI* restriction site was introduced at nucleotide 72 of the open reading frame of *GPI8.* PCR fragment 1 (primers G8NterF 5′-gagcaacaaatggaattagcc-3′ and G8BsiWINR 5′-agcatcc*gtacg*gttatttgc-3′) was digested with *XhoI* and *BsiWI*, and PCR fragment 2 (G8BsiWINF 5′-gcaaataacc*gtacg*gatgct-3′ and G8NterR 5′-ggaatacattgtgtttgcctg-3′) were digested with *BsiWI* and *BsmI* and were ligated into pBF53 (Meyer *et al.*, 2000), which was digested with *XhoI* and *BsmI*. GST (EC 2.5.1.18) from *Schistosoma japonicum* was amplified with the primers GSTBsiWIF 5′-ggct*cgtacg*atgtcccctatactaggt-3′ and GSTBsiWIR 5′-ggct*cgtacg*atccgattttggaggatg-3′, each of which contained a *BsiWI* restriction site. The GST fragment was digested with *BsiWI* and was introduced into the *BsiWI* site in *GPI8*.

Preparation of Microsomal Membranes and Solubilization of Membrane Proteins

Microsomal membranes were prepared as previously described (Reiss *et al.*, 1997) with the following modifications: cells were grown at 37°C, and membranes were frozen at a higher concentration (0.2 ml membrane buffer was added to the membrane pellet from 1′000 OD_{600} units of cells). After thawing, 300 μl TM buffer (50 mM Tris-HCl pH7.4, 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 plus the protease inhibitor mix described for membrane buffer) 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 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 × *g*) at 4°C in a TFT 80.4 fixed angle rotor (Kontron, Munich, Germany). The concentration of solubilized proteins was determined and samples were snap frozen in liquid nitrogen and stored at −80°C.

Blue Native PAGE

Buffers and gel composition were used as previously described (Schägger and von Jagow, 1991; Schägger, 1995), but Tris-HCl, adjusted to pH 7.5 at 4°C, was used instead of Bistris. Solubilized proteins were adjusted to 1 μg/μl with GTM buffer containing 525 mM 6-aminocaproic acid, 1.5% digitonin, 1 mM DTT, 1 mM PMSF, and the described protease inhibitor mix. A 0.15 volume of sample buffer was added, samples were mixed gently and were loaded onto 5–15% or 5–12% polyacrylamide gradient gels. The electrophoresis was carried out at 4°C; after one third of the running time, the cathode buffer with SERVA-Blue was removed and replaced by a cathode buffer without dye. Western blots were revealed with the

use of the chemiluminescence ECL kit from Amersham Pharmacia (Uppsala, Sweden).

Affinity Chromatography of GST-Gpi8p

All procedures were carried out at 4°C. Microsomes from 4000 OD_{600} units of FBY656 cells were prepared and solubilized as for blue native gel electrophoresis but without the use of 6-aminocaproic acid. The lysate was diluted to 10 ml with TM buffer and was mixed with 1 ml glutathione-Sepharose 4B. Beads were incubated overnight at 4°C on a wheel. Beads were sedimented by letting them stand for 1 h at 4°C, and the supernatant was carefully removed (unbound fraction, see Figure 2). The glutathione-Sepharose was washed three times, each time adding 10 ml TM buffer + 0.3% digitonin, rotating the tube on the wheel for 15 min, and letting it stand for 1 h. The supernatants were carefully removed (washes 1 to 3, Figure 2). The bound protein was eluted by adding 1 ml TM buffer plus 0.3% digitonin plus 20 mM reduced glutathione (Sigma, St. Louis, MO), and gently rotating the tube for 30 min. The supernatant was decanted carefully (eluate E20) and was concentrated by ultrafiltration with the use of a Centricon 3KD centrifugation device (Millipore Corp., Bedford, MA) at 4°C, 7000 × *g* for 2 h. This elution process was repeated by adding 1 ml of TM buffer plus 0.3% digitonin plus 100 mM reduced glutathione, pH 8.0. The supernatant was decanted and concentrated (eluate E100).

Tryptic Digestion and Mass Spectrometry of Proteins

The preparative SDS-PAGE gel (Figure 2) was stained with silver nitrate, and the interesting bands were cut out and cut into small pieces with a scalpel. Then, 100 μl of 25 mM NH_4HCO_3 were added, gel pieces were agitated with a Vortex for 8 min, and the supernatant was discarded. Washing by agitation was reported using 100 μl of acetonitrile. Alternating washes with these two solvents were repeated two more times. Gel pieces were dried completely with a SpeedVac evaporator before reduction and alkylation. For this, the gel pieces were covered with 100 μl of 10 mM DTT in 25 mM NH_4HCO_3 and were left at 57°C for 1 h. The supernatant was removed, 100 μl of 55 mM iodoacetamide in 25 mM NH_4HCO_3 were added, and gel pieces were left in the dark at room temperature for 1 h. The supernatant was removed, and the gel pieces were washed three times with 100 μl of NH_4HCO_3 and three times with acetonitrile, as above. Gel pieces were dried completely in the SpeedVac evaporator before tryptic digestion. The dried gel volume was evaluated, and three volumes of freshly diluted trypsin (12.5 ng/μl) in 25 mM NH_4HCO_3 were added. The digestion was performed at 35°C overnight. Then, 5 μl of 25% H_2O/70% acetonitrile/5% HCOOH were added, and the samples were sonicated for 5 min and centrifuged. The supernatant was recovered, and the elution of peptides from gel was repeated once using the same solvent. The volume of the supernatant was reduced under a N_2 flow to 4 μl, 1 μl of H_2O/5% HCOOH was added, and 0.5 μl was used for matrix-assisted laser-desorption time-of-flight (MALDI-TOF) analysis.

Mass measurements were carried out on a Bruker BIFLEX MALDI-TOF mass spectrometer equipped with SCOUT high-resolution optics with X-Y multisample probe and gridless reflector. This instrument was used at a maximum accelerating potential of 20 kV and was operated in reflector mode. Ionization was accomplished with a 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 1 GHz. A saturated solution of α–cyano-4-hydroxycinnamic acid in acetone was used as a matrix. A first layer of fine matrix crystals was obtained by the spreading and fast evaporation of 0.5 μl of matrix solution. Subsequently, a droplet of 0.5 μl of aqueous HCOOH (5%) solution was deposited onto this fine layer of crystals. Thereafter, 0.5 μl of sample solution was added, and a second droplet of 0.2 μl of the matrix-saturated solution of 50% H_2O/50% acetonitrile was added. The preparation was dried in a vacuum. The sample was washed one to three times by applying 1 μl of aqueous HCOOH (5%) solution on the target and then blowing it off after a few seconds. The calibration was performed in internal mode with the following four peptides: angiotensin (1046.542 Da); substance P (1347.736 Da); bombesin (1620.807 Da); and ACTH (2465.199 Da).

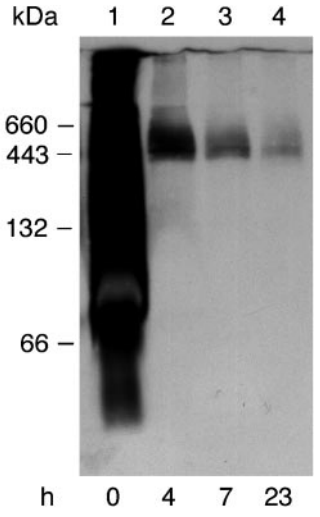


Figure 1. Blue native PAGE of microsomal membrane proteins during Gpi8p depletion. FBY164 (*gpi8::kanMX2 ura3–1:: URA3-GAL1,10-GPI8*) cells were grown overnight at 24°C in SGaa medium, were shifted to SDaa medium, and were incubated for 0, 4, 7, and 23 h at 37°C. After incubation, the cells were harvested, microsomes were prepared, and membrane proteins were solubilized in digitonin and processed for blue native PAGE. Solubilized proteins (40 μg per lane) were loaded on a 5–15% polyacrylamide gradient gel. Proteins were transferred onto a PVDF membrane and probed with antibodies against Gpi8p. Marker proteins were bovine serum albumin (monomeric and dimeric form, 66 and 132 kDa, respectively), apoferritin (443 kDa), and thyroglobulin (660 kDa).

Other Methods

The membrane association, N-glycosylation, and membrane orientation of Gpi16p were investigated as described previously (Benghezal *et al.*, 1996). Cells were labeled with [2-^{3}H]-*myo*-inositol, and lipid extracts were analyzed by TLC as previously described (Meyer *et al.*, 2000). All SDS-PAGE procedures were done under reducing conditions.

RESULTS

Gpi8p Is Stabilized by Its Integration into a High-Molecular-Weight Complex

To investigate the interaction of Gpi8p with potential partners under physiological conditions, we ran digitonin extracts of microsomes in blue native gel electrophoresis. This method allows the preservation of the tertiary and quaternary structures of protein complexes but, nevertheless, allows the separation of the complexes according to their molecular weight (Schägger and von Jagow, 1991; Schägger, 1995). Cells lacking the wt *GPI8* but expressing wt Gpi8p under the control of a *GAL1,10* promoter were grown on galactose and then shifted to glucose. A previous study

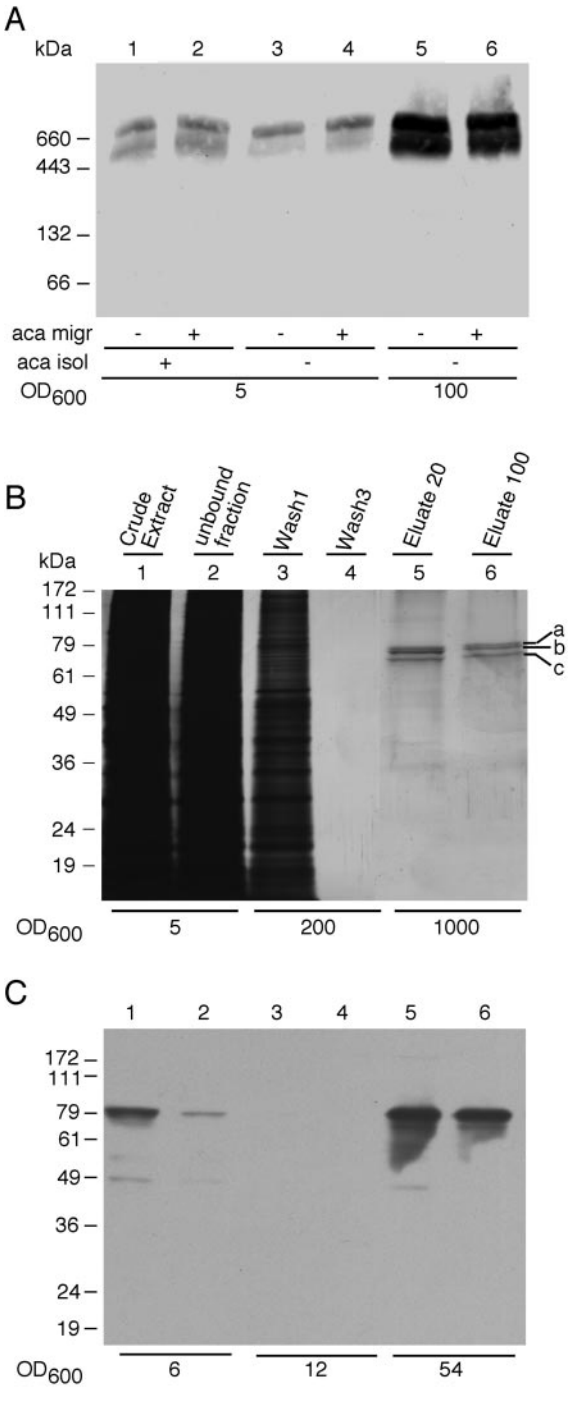
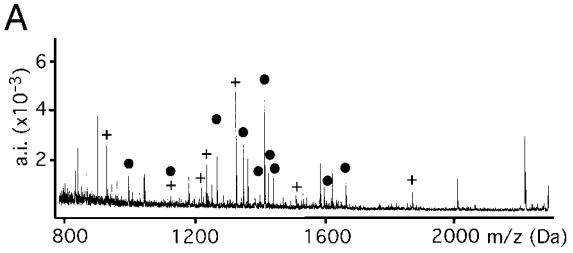


Figure 2. Affinity chromatography purification of a multiprotein complex containing the GST-tagged form of Gpi8p. (A) Membranes of FBY656 cells (*gpi8*Δ GST-Gpi8p) were isolated and solubilized either in the presence (+) or absence (-) of 6-aminocaproic acid (aca isol). The bulk of the lysate prepared without 6-aminocaproic acid was subjected to affinity chromatography on glutathione-Sepharose 4B. Solubilized proteins were prepared for electrophoresis in the loading buffer with (+) or without (-) 6-aminocaproic acid (aca migr). Aliquots from extracts (lanes 1–4) and from affinity-purified protein (lanes 5 and 6) were separated on a 5–12% blue native gel. Gpi8p was detected by Western blotting with anti-Gpi8p antibody. (B) Material bound to glutathione-Sepharose was eluted with the use of 20 mM, then 100 mM, reduced glutathione (eluates 20 and 100, respectively). The crude extract (lane 1), material not binding to the Sepharose (lane 2), washes 1 and 3 (lanes 3 and 4), and eluates (lanes 5 and 6) were loaded onto a 12% polyacrylamide gel for SDS-PAGE. The gel was stained with silver nitrate. (C) The presence of the GST-tagged form of Gpi8p in the eluates was confirmed by probing a Western blot of a parallel gel containing the same fractions with anti-Gpi8p antibodies. The amounts of material loaded onto the gels are indicated in OD_{600} equivalents.

(Meyer *et al.*, 2000) demonstrated that under these conditions the very same cells massively overexpress Gpi8p on galactose and reach wt levels of Gpi8p after 4.5 h of growth on glucose. As shown in Figure 1, material reacting with the anti-Gpi8p antibody is found as a broad smear covering the whole range of molecular weights from the top of the stacking gel down to a major band corresponding to ~65–85 kDa. (In SDS-PAGE, the three glycoforms of Gpi8p have apparent molecular masses of 50, 48, and 46 kDa (Benghezal *et al.*, 1996).) After 4 h on glucose, the amount of Gpi8p is drastically reduced, and almost all Gpi8p is found within high-molecular-weight bands corresponding to apparent molecular masses of 430–650 kDa. The large smear observed in cells overexpressing Gpi8p may represent Gpi8p interacting with numerous other proteins and/or with itself. The result strongly suggests that under physiological conditions (Figure 1, lane 2) Gpi8p is part of a defined stoichiometric protein complex and does not exist as a monomer. We previously reported (Meyer *et al.*, 2000) that in the very same type of experiment, where one goes from overexpression to depletion, turnover of Gpi8p is at least four times faster in the period when Gpi8p is above physiological levels than afterward. The data in Figure 1 demonstrate that it is the Gpi8p fraction, which resides outside the high-molecular-weight complexes, that turns over rapidly, whereas the Gpi8p within these complexes turns over more slowly.

The Purified GPI Transamidase Complex Contains Gaa1p, Gpi8p, and a Novel Component, Gpi16p

The GPI transamidase complex was isolated by affinity chromatography over glutathione-Sepharose from the digitonin extract of microsomes prepared from cells containing GST-tagged Gpi8p rather than wt Gpi8p. The original protocol of blue native gel electrophoresis utilizes high concentrations of the detergent-like molecule 6-aminocaproic acid in addition to digitonin for solubilization and during electrophoresis. Our preliminary experiments showed that 6-aminocaproic acid increased the efficiency of extraction but that even without it the same 430–650-kDa complex was obtained. The same is shown in Figure 2A, lanes 1–4, although in this case the complex is bigger (490–780 kDa), since Gpi8p was tagged with GST. This suggested that the size of this complex is dictated by the inherent binding properties and interactions of its components rather than by the solubilization conditions. For large-scale purification of the complex, 6-aminocaproic acid was left out. The SDS-PAGE profile of the proteins present at the different purification steps is shown in Figure 2B. GST-Gpi8p was absorbed nearly quantitatively and could be eluted by free glutathione (Figure 2C, lanes 1, 2, 5, and 6). The complex was eluted in intact form,

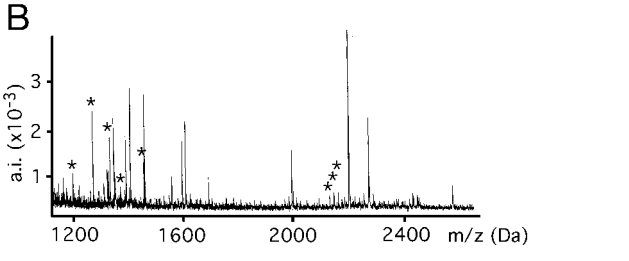


M/z Submitted	MH+ matched	Start	end	Peptide Sequence
932.4458	932.4419	48	53	FWFNYR
1127.5803	1127.5485	132	140	WTEDHPKSK
1221.5680	1221.5873	54	63	HMANVLSMYR
1237.5410	1237.5822	54	63	HMANVLSMYR 1Met-ox
1326.6298	1326.6694	116	126	GYEVTVENFIR
1510.7496	1510.7654	127	138	LLTDRWTEDHPK
1866.8477	1866.8510	100	115	DHAIDLYGDSVEVDYR
2616.2850	2616.2292	142	164	LLTDENSNIFIYMTGHG GDDFLK 1Met-ox

Yeast GPI8 Protein. DB Accession No.: P49018

999.5000	999.4900	462	469	YYLQAADR
1127.5803	1127.6061	219	229	SGLTSLLDGHK
1127.5803	1127.5849	462	470	YYLQAADRK
1268.7059	1268.7214	84	95	AIPPLLESTATR
1348.7034	1348.6392	400	412	SLTGNGQDRGGMR
1398.6519	1398.7018	413	424	IVFHNDNDTPVK
1413.8208	1413.8582	387	399	VTPIVPVPIHVSR
1423.7258	1423.7731	262	274	IEMVTHVPSALAR
1439.7159	1439.7681	262	274	IEMVTHVPSALAR 1Met-ox
1617.7350	1617.8139	425	436	LIYFESLPWFMR 1Met-ox
1662.8974	1662.9291	275	289	GERPIPKPLDGNTLR

Yeast GPI16 Protein. DB Accession No.: P38875



1214.6493	1214.6533	439	448	IHISEPLSYR
1287.6803	1287.7061	172	182	NIIVVFSENPR
1342.7076	1342.7483	438	448	KIHISEPLSYR
1385.6073	1385.6715	288	299	KPHGNEAFSGWR
1469.6540	1469.6886	307	320	AHGNSGHDITTFGR
2140.9048	2141.0014	51	68	TYISENALMPSQAYSYFR
2156.9664	2156.9963	51	68	TYISENALMPSQAYSYFR 1Met-ox
2173.0471	2173.0624	487	506	TIVESSSEHEVTTQSSNPIK

Yeast GAA1 Protein. DB Accession No.: P39012

Figure 3. Mass spectrometric identification of Gpi8p-associated proteins. (A) Spectrogram of peptide masses obtained from tryptic digestion of bands a and b (analyzed jointly) from the gel shown in Figure 2B, lanes 5 and 6. Peptide masses were used to query an amino acid sequence database (PROTEIN PROSPECTOR). Proteins were identified by correlating the measured peptide masses to a theoretical tryptic digest of all proteins present in the database. Yeast Gpi8p and Gpi16p were the only yeast proteins that were identified. Their peptides are marked with crosses (+, Gpi8p) and dots (●, Gpi16p). (B) Spectrogram of peptide masses obtained from band c (Figure 2B) identified Gaa1p (*). The exact tryptic peptides allowing identification are listed. a.i. = absolute intensity.

since when run side by side with the starting material in blue native gel electrophoresis, the purified complex had the same mobility as the complex present in the original microsomal extract (Figure 2A, lanes 1–4 vs. lanes 5 and 6). Thus, no major loss of subunits seems to occur during affinity chromatography. By silver nitrate staining it was revealed that the eluted complex contained only three distinct bands,

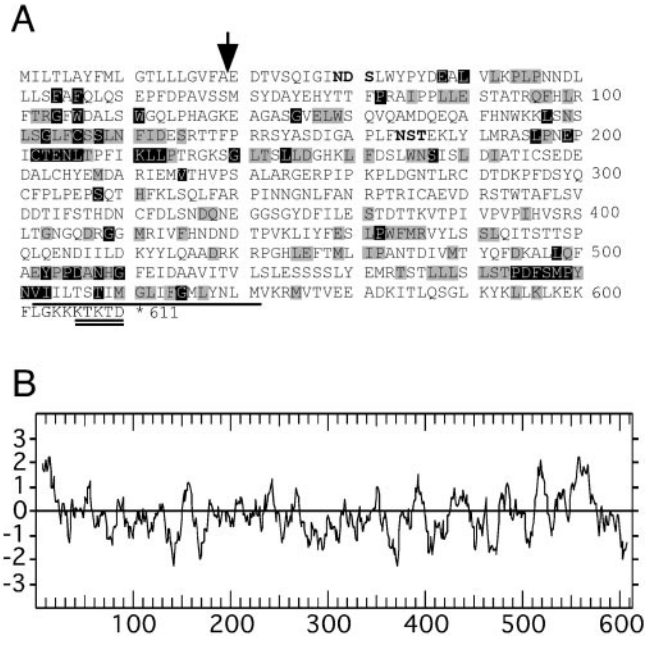


Figure 4. Sequence of *S. cerevisiae* Gpi16p. (A) Potential N-glycosylation sites are shown in boldface, the C-terminal TMD is underlined, and the ER retrieval domain doubly underlined. The most likely cleavage site of the signal peptidase is indicated by a vertical arrow. The Gpi16p homologues of *Schizosaccharomyces pombe* (emb-CAA22348.1), *Drosophila melanogaster* (gb AAF46367.1), *Homo sapiens* (CGI-06 protein, XM 009533.2), *Caenorhabditis elegans* (emb-CAA96629.1), and *Arabidopsis thaliana* (gb AAF20232.1) were aligned usig the CLUSTALW multiple sequence alignments program 1.81. Only the yeast Gpi16p sequence is shown, but identities and conservative substitutions are represented by black and gray boxes. (B) A hydropathy plot, drawn according to Kyte and Doolittle, is shown in which the hydrophobic sequences get a positive score.

among which GST-Gpi8p was detectable by Western blotting (Figure 2C, lanes 5 and 6). These bands were cut out and were further analyzed by tryptic digestion and MALDI-TOF mass spectrometry. As shown in Figure 3, only three yeast proteins could be identified by this procedure. Bands a and b of Figure 2B, lanes 5 and 6, which had been excised from the gel en bloc, contained tryptic fragments of GST-Gpi8p and YHR188c, and band c contained fragments of Gaa1p. Indeed, the genes for these three proteins predict translation products of 635, 610, and 614 amino acids. Gaa1p has been described to have an apparent mass of 70 kDa in SDS-PAGE (Hamburger *et al.*, 1995).

The sequence and the hydrophobicity plot of YHR188c, henceforth named *GPI16*, are shown in Figure 4. Two versions of Gpi16p differing by the presence or absence of eight hydrophobic residues at the N-terminus are currently found in genomic databases, the shorter version being proposed based on the sequence submission of C. Marci to the EMBL Data Library in 1994 (entry S46687), and the longer one based on the complete sequence of chromosome VIII submitted by Johnston *et al.* (1994). Both versions predict a type I membrane protein with an N-terminal signal sequence for insertion into the ER, a C-terminal TMD, and an ER retrieval

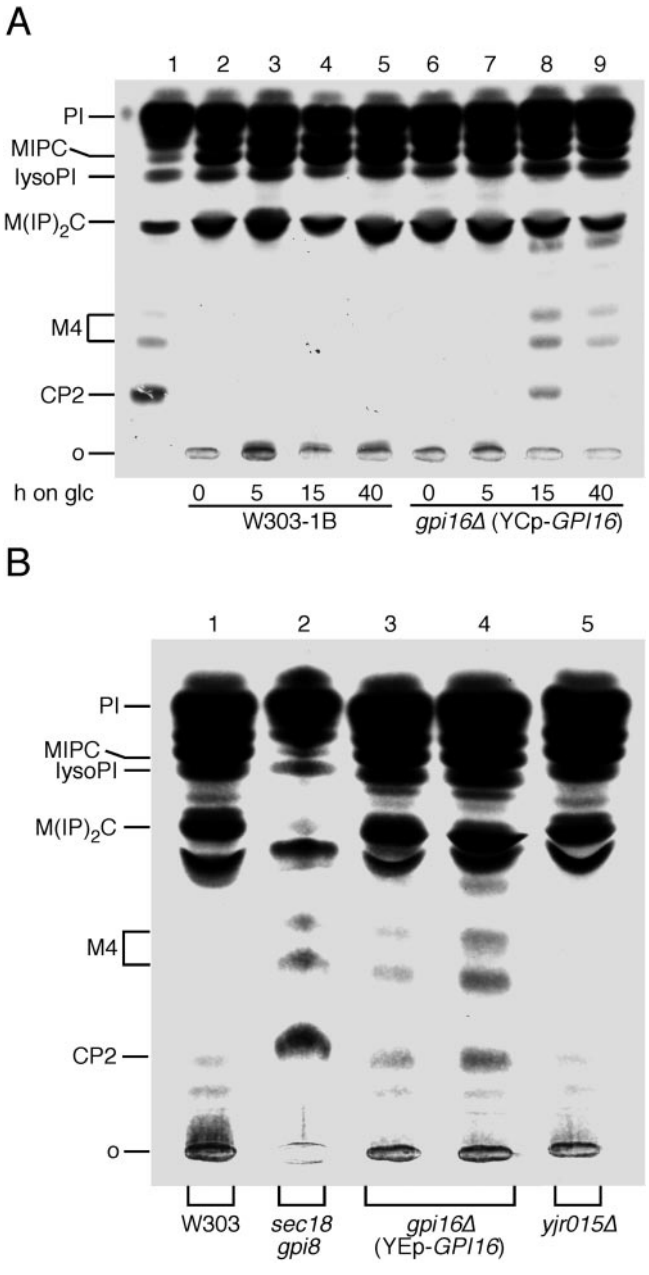


Figure 5. Cells depleted of Gpi16p accumulate complete GPI lipids. (A) W303 and FBY735 (*gpi16*Δ YCplac111-*GAL1-GPI16*) were grown to exponential phase at 30°C in SGaa. The expression of Gpi16p was subsequently blocked by shifting cells to glucose medium (SDaa). Cultures were continued at 30°C for 0, 5, 15, or 40 h and were periodically diluted with fresh medium to keep the cell concentration in the range of 0.5–2.5 OD_{600}. The *sec18 gpi8* cells (lane 1) were grown at 24°C and were preincubated for 5 min at 37°C. Aliquots of cells were radiolabeled at 37°C with [2-^{3}H]-*myo*-inositol for 80 min, and lipid extracts were analyzed by TLC. (The *sec18 gpi8* double mutant was chosen as a positive control, since it accumulates more CP2 at 37°C than *gpi8*.) (B) FBY733b cells (*gpi16*Δ pYES2-*GPI16*) were grown to exponential phase at 30°C in SGaa medium and were shifted to SDaa for 2 h (lane 3) or 15 h (lane 4). W303 cells and *yjr015*Δ cells were grown at 30°C. Aliquots of cells were radiolabeled and processed as above.

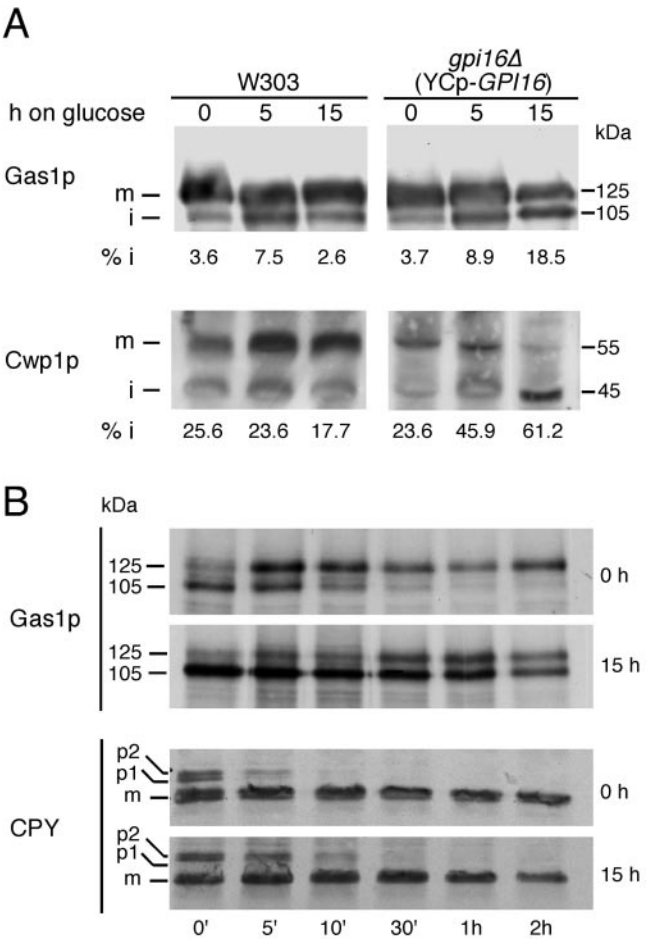


Figure 6. Depletion of Gpi16p blocks maturation of Gas1p and Cwp1p. (A) W303–1B and FBY735 cells (*gpi16*Δ YCp-*GAL1-GPI16*) were grown to exponential phase at 30°C in SGaa medium and were shifted to glucose for 0, 5, and 15 h in order to block the expression of Gpi16p. Proteins were extracted, separated by SDS-PAGE, Western blotted, and probed with anti-Gas1p and anti-Cwp1p antibodies. The relative amounts of the immature 105-kDa form of Gas1p as well as of the immature 45-kDa form of Cwp1p were determined by densitometry and are given as the percentage of total Gas1p or Cwp1p in the extracts. m = mature; i = immature. (B) FBY735 cells were grown to exponential phase at 30°C in selective SGaa medium, were washed twice in distilled water, and were resuspended in SDaa medium. After 0 or 15 h of culture, cells were pulse-labeled with [^{35}S]-methionine and [^{35}S]-cysteine for 10 min and were chased for the indicated times. Gas1p was immunoprecipitated from the lysates and analyzed by SDS-PAGE and fluorography.

motif at the end of the cytosolic C-terminus. Gpi16p is a protein of unknown function that has homologues in humans, flies, worms, plants, and other fungi. These organisms contain proteins of similar size (531–639 amino acids) and a similar hydropathy profile. When comparing the proteins over their entire length, these proteins (specified in the legend of Figure 4A) exhibit 24–32% identities and 43–49% similarities to *GPI16*. Identities are concentrated into several motifs appearing in yeast Gpi16p at residues 147–163, 196–213, 498–510, and 544–565 (Figure 4A). Only the yeast mem-

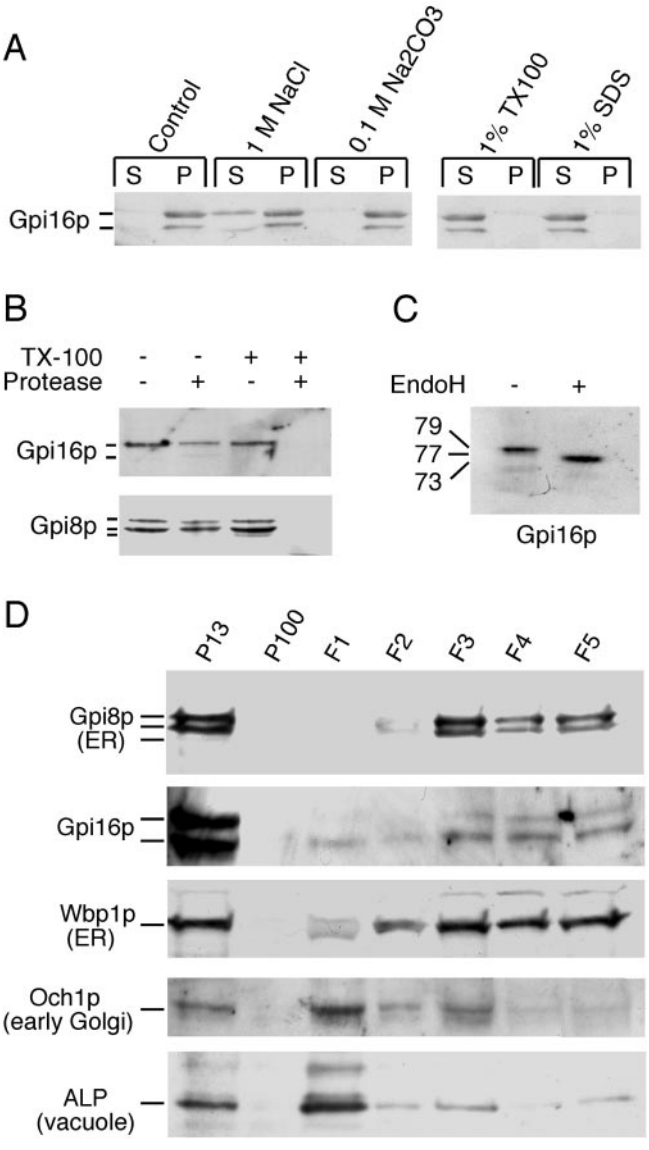


Figure 7. Gpi16p is a lumenally oriented, integral membrane glycoprotein of the ER. (A) Exponentially growing W303–1B were broken with glass beads and cell walls were removed. Aliquots of the lysate (equivalent to 20 OD_{600}) were incubated for 30 min at 0°C with NaCl, Na_2CO_3, Triton X-100, or SDS. Subsequently, membranes were sedimented by ultracentrifugation, proteins of supernatants (S) and pellets (P) were precipitated with TCA, were processed for SDS-PAGE and Western blotting, and were probed with affinity-purified rabbit anti-Gpi16p antibodies. (B) Microsomes from W303–1B were digested with 0.1 mg/ml proteinase K at 0°C for 30 min in the presence or absence of 0.5% of Triton X-100. Samples were resuspended in sample buffer, processed for Western blotting, and probed with anti-Gpi16p or anti-Gpi8p antibodies. (C) A microsomal pellet from W303–1B cells was solubilized in 1% SDS and was incubated with or without 1 mU/OD_{600} endoglycosidase H for 20 h at 37°C. The samples were processed as above. (D) Cellular localization of Gpi16p was determined by subcellular fractionation. For this experiment, a slightly modified version of a published procedure (Gaynor *et al.*, 1994) was used. Spheroplasts of W303–1B at 67 OD_{600}/ml were osmotically lysed in a syringe, the pellet P13 obtained through centrifugation at 13,000 × *g* for 15 min was further resolved by sedimenting 100-OD_{600} equivalents through a step gradient of 1.6 ml 1.2 M sucrose to >1.6 ml 1.5 M sucrose, yielding 5 0.7-ml fractions termed F1 to F5. Supernatant S13 was further centrifuged at 100,000 × *g* for 1 h to obtain pellet P100. Equivalent aliquots of all fractions were processed for SDS-PAGE and Western blotting. On prolonged incubation of microsomes, variable amounts of a 69-kDa degradation product of Gpi16p was generated.

bers have a C-terminal ER retrieval motif, but the proteins of other species may be retained in the ER by other signals or by interaction with Gaa1p or Gpi8p.

Gpi16p Is Essential for GPI Anchoring

YHR188c has been found to be an essential gene. We therefore generated haploid *gpi16*Δ strains that contain the open reading frame of *GPI16* behind the *GAL1* promoter, either on a multicopy or a centromeric vector. Western blot analysis with the use of a rabbit antibody to an immunogenic peptide of Gpi16p demonstrated that Gpi16p could be significantly depleted when cells were shifted from a galactose- to a glucose-containing medium (see below). When Gpi16p-depleted cells were labeled with [^{3}H]-*myo*-inositol, cells accumulated the GPI precursor lipids CP2 and two species of M4 (Figure 5A, lane 8, and B, lanes 3 and 4). These rather mature GPI precursor lipids are not normally present in the lipid extract of wt cells (Figure 5A, lanes 2–5, B, lane 1), but only appear if the GPI transamidation reaction is blocked, e.g., in *gpi8* (Figure 5A, lane 1, B, lane 2). When Gpi16p is depleted for longer periods, the cells start to grow faster again and the accumulation of GPI lipids is less pronounced (Figure 5A, lane 9), indicating that cells may undergo some genetic or metabolic adaptation, possibly by increasing the number of plasmids carrying *GPI16*.

The accumulation of immature ER forms of GPI proteins such as Gas1p (Nuoffer *et al.*, 1991) or Cwp1p (Shimoi *et al.*, 1995) is a symptom of a delay or deficiency in the GPI anchor addition to newly synthesized proteins (Doering and Schekman, 1996). Western blotting shows an accumulation of the immature 105-kDa form of Gas1p and the immature 45-kDa form of Cwp1p after 5 and 15 h of depletion of Gpi16p (Figure 6A), whereas there was no accumulation of immature carboxypeptidase Y (CPY) (not shown). In pulse-chase experiments, it appears that the maturation of Gas1p from the 105-kDa immature form to the 125-kDa mature form is severely delayed when Gpi16p is depleted, whereas the maturation of CPY is not significantly retarded (Figure 6B). Thus, it appears that the depletion of Gpi16p induces a specific defect in (with regard to) the attachment of GPI lipids to newly made GPI proteins and by consequence, delays their transport to the Golgi.

A systematic two-hybrid screen had detected a significant interaction of Gpi16p with YJR015w (Uetz *et al.*, 2000), a nonessential protein of 510 amino acids having several TMDs. As seen in Figure 5B, lane 5, *yjr015w*Δ cells did not accumulate any GPI precursor lipids. Further studies are required to definitely rule out a role for this protein in GPI anchor addition.

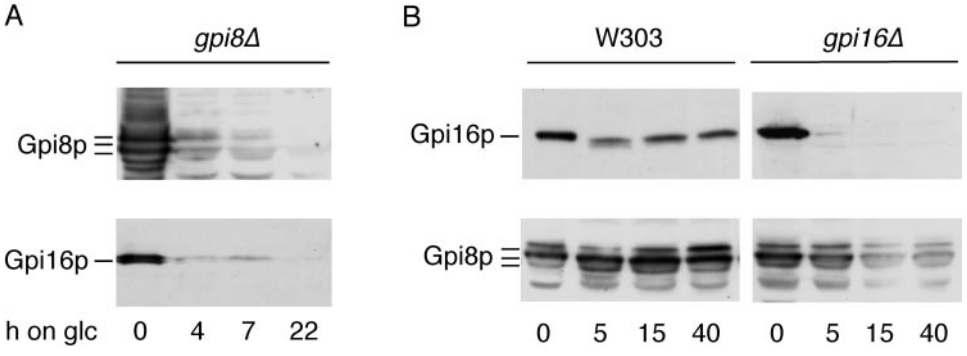


Figure 8. Gpi8p and Gpi16p are mutually stabilizing each other. FBY164 (*gpi8::kanMX2 ura3–1::URA3-GAL1,10-GPI8*), W303, and FBY735 (*YHR188c::kanMX4* containing plasmid YCplac111-*GAL1-GPI16*) were grown to exponential phase at 30°C in galactose medium (SGaa). The expression of Gpi8p (FBY164) or Gpi16p (FBY735) was subsequently blocked by shifting cells to glucose medium (SDaa). Cultures were continued at 30°C for 0, 4, 7, and 22 h (FBY164) or for 0, 5, 15, and 40 h (W303 and FBY735). Proteins were extracted, separated by SDS-PAGE, Western blotted, and probed with anti-Gpi8p (A) and anti-Gpi16p antibodies (B); membranes were stripped and probed again with anti-Gpi16p (A) and anti-Gpi8p (B) antibodies.

Gpi16p Is an Integral Membrane Glycoprotein of the ER

High salt or high pH treatment of microsomal membranes cannot remove Gpi16p from the membranes (Figure 7A), but the protein is rendered soluble by Triton X-100 at 4°C. Protease protection experiments on microsomes from wt cells indicate that Gpi16p is protease-resistant unless detergent is added in the same way as with Gpi8p (Figure 7B). The treatment of the cell lysate with endoglycosidase H shifted the molecular mass of Gpi16p by ~6 kDa (Figure 7C), thus indicating that probably both of the two potential N-glycosylation sites are utilized. Subcellular fractionation indicates that Gpi16p is mainly present in fractions that contain the ER markers Gpi8p and Wbp1p, and is absent from lighter fractions that are enriched in the Golgi marker Och1p and the vacuolar alkaline phosphatase (ALP) (Figure 7D). These data indicate that Gpi16p is an integral ER membrane glycoprotein, with the bulk of the protein being oriented toward the lumen of the ER. It probably gets directed into the ER by its N-terminal signal sequence.

Gpi8p and Gpi16p Are Mutually Stabilizing Each Other

Figure 1 suggests that Gpi8p, if not integrated into the transamidase complex, is unstable. Since Gpi16p is a major component of this complex, we expect that the depletion of Gpi16p would lead to a depletion of Gpi8p. As shown in Figure 8B, this is indeed the case. When interpreting this figure, one has to keep in mind that at time 0 the cells strongly overexpress Gpi16p but contain physiological levels of Gpi8p. Thus, even after 40 h of depletion, the remaining Gpi16 and Gpi8p may still be present at a physiological ratio. Previous experiments have shown that during 24 h on glucose the Gpi8p content in the *gpi8*Δ cells used in Figure 8A is dropping from 1140% to <6% of the physiological amount of Gpi8p (Meyer *et al.*, 2000). Figure 8A demonstrates that the depletion of Gpi8p in these cells leads to a concomitant loss of Gpi16p. This suggests that Gpi16p, in a manner similar to that of Gpi8p, is unstable unless integrated into the transamidase complex. The experiment shown in Figure 8B raised the possibility that the GPI-anchoring deficiency observed in Gpi16p-depleted cells may be due to the concomitant depletion of Gpi8p, which is believed to be the catalytic subunit of the transamidase complex. Thus, it was conceivable that Gpi16p affected the GPI anchor addition solely through the stabilization of Gpi8p. However, as shown in Figure 9, the overexpression of Gpi8p could not rescue the growth phenotype of Gpi16p-depleted cells. The same result also was obtained in liquid cultures (not shown). Moreover, the same cells used for the blotting experiment of Figure 9B were also metabolically labeled with [^{3}H]-*myo*-inositol, and their lipid extracts were analyzed by TLC as described in Figure 5. This showed that the accumulation of the abnormal GPI lipids CP2 and M4 caused by the depletion of Gpi16p (Figure 5) could not be hindered or attenuated by the overexpression of Gpi8p (not shown). These data suggest that Gpi16p, besides stabilizing Gpi8p, also has a more direct effect on the GPI anchor addition.

The GPI Transamidase Complex Is not Associated with Sec61p or Wbp1p

The major components of the 430–650-kDa transamidase complex are Gaa1p, Gpi8p, and Gpi16p, but it seemed conceivable that before blue native gel electrophoresis, in the digitonin extract, this complex was associated with other protein complexes involved in the processing of GPI proteins. Some kind of association of the transamidase complex with the ER translocation pore is suggested by the fact that all current in vitro assays for the GPI anchor addition are combined translocation/GPI attachment assays, and all our attempts to assay the transamidase with artificial peptides that do not need to be translocated have failed so far (P. Fraering, unpublished data). Therefore, we tried to evaluate whether the transamidase complex is associated with the ER translocation pore and the oligosaccharyltransferase complex. The latter may be in close vicinity to the translocation pore because proteins can be cotranslationally N-glycosylated. The yeast translocation complex is composed of two subcomplexes, the Sec61 complex, consisting of Sec61p (41 kDa), Sss1p (9.1 kDa), and Sbh1p (8.7 kDa), and the Sec62/63 complex, consisting of Sec62p (30 kDa), Sec63 (73 kDa), Sec71p (31 kDa), and Sec72p (23 kDa). The Sec61 complex is competent for the cotranslational translocation of proteins, whereas the presence of both subcomplexes is required for posttranslational translocation (Ng *et al.*, 1996). The oligosaccharyltransferase activity depends on Ost1p (64 kDa),

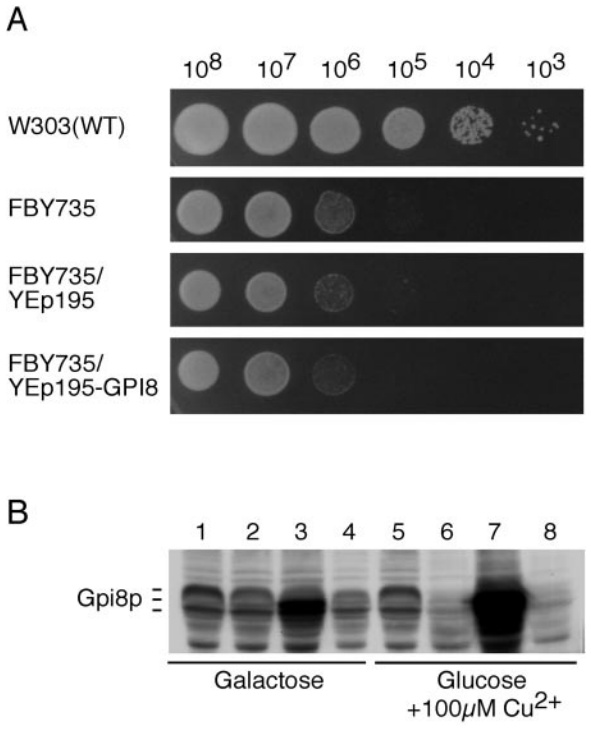


Figure 9. Overexpression of Gpi8p cannot suppress the growth defect of Gpi16p-depleted cells. (A) W303–1B (wt), FBY735 *(Δgpi16/* YCplac111-*GAL1-GPI16*), FBY735 transfected either with empty YEplac195, or with YEplac195 harboring *GPI8* placed under the control of the promoter of *CUP1* (YEplac195-*CUP1-GPI8*) were grown to exponential phase at 30°C in galactose-containing medium. To deplete them of Gpi16p and to induce Gpi8p, 3-μl aliquots of cells at concentrations shown at the top (cells/ml) were inoculated onto plates containing glucose plus 100 μM Cu^{2+}. The plates were incubated for 35 h at 30°C before being photographed. (B) W303 (lanes 1 and 5), FBY735 (lanes 2 and 6), FBY 735 transfected with YEplac195-*CUP1-GPI8* (lanes 3 and 7), and FBY735 transfected with the empty vector YEplac195 (lanes 4 and 8) were grown at 30°C for 15 h in liquid culture with the use of either galactose medium or, alternatively, glucose medium supplemented with 100 μM Cu^{2+}. Proteins were extracted, separated by SDS-PAGE, Western blotted, and probed with anti-Gpi8p antibodies.

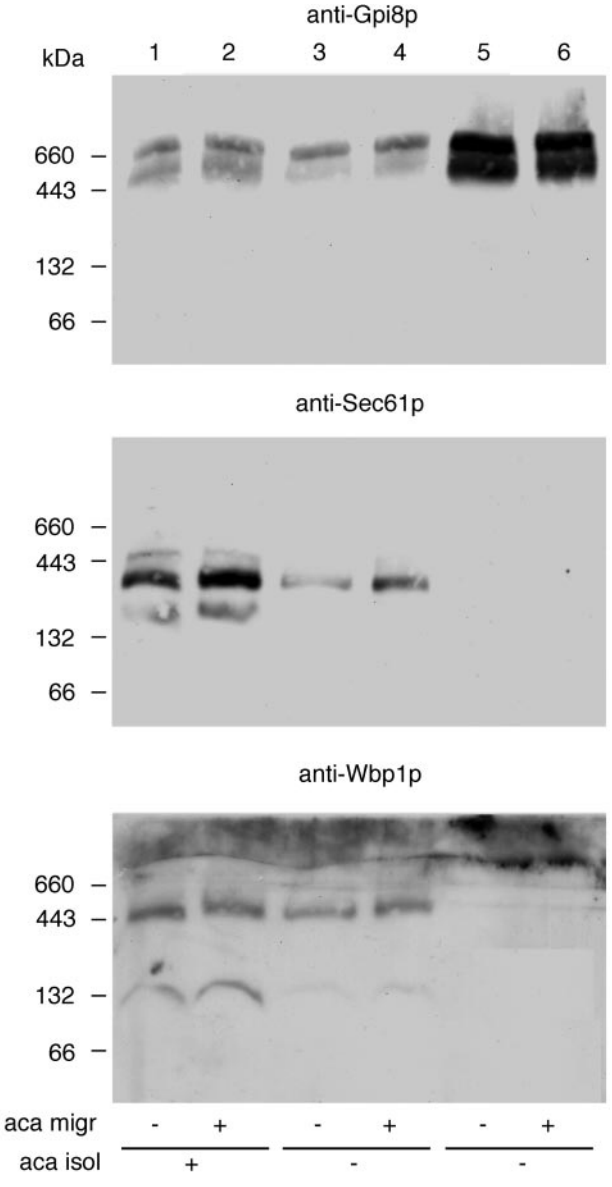


Figure 10. Association of the transamidase complex with other multimeric ER complexes. The membrane of the Western blot shown in Figure 2A was stripped and probed again with anti-Sec61p antibodies, was stripped again, and finally was probed with anti-Wbp1p antibodies. The top panel is the same as in Figure 2A.

Wbp1p (45 kDa), Swp1p (30 kDa), Ost2p (16 kDa), Ost3p (34 kDa), Stt3p (78 kDa), Ost4p (3.4 kDa), Ost5p (9.5 kDa), and Ost6p (37.3 kDa) (Knauer and Lehle, 1999). The sum of these subunits amounts to 317.6 kDa. In blue native gel electrophoresis, the oligosaccharyltransferase migrated as a 240-kDa complex that, as determined by Western blot analysis, contained all of these subunits (Knauer and Lehle, 1999). Each individual complex may, however, be composed of only a subset of components since some subunits may be redundant. As shown in Figure 10, when analyzed by blue native gel electrophoresis of digitonin extracts, Sec61p and Wbp1p do not exist in free form but are present mainly in high-molecular-weight complexes of 180, 310, and 470 kDa (Sec61p) and 440 kDa (Wbp1p), respectively. In this experiment, the cells contained GST-tagged Gpi8p rather than wt Gpi8p, so that the GPI transamidase complex could be isolated by affinity chromatography over glutathione-Sepharose. The purified transamidase complex contained neither Sec61p nor Wbp1p (lanes 5 and 6). Overall, the data indicate that an association of the GPI transamidase complex with these other complexes either does not exist or does not survive digitonin extraction and affinity purification. The lower mass estimation for the oligosaccharyltransferase complex obtained by Knauer and Lehle (1999) may be due to the fact that they used Nikkol plus 6-aminocaproic acid as detergents, whereas we used digitonin. Incidentally the data show that 6-aminocaproic acid is not absolutely required for solubiliza-

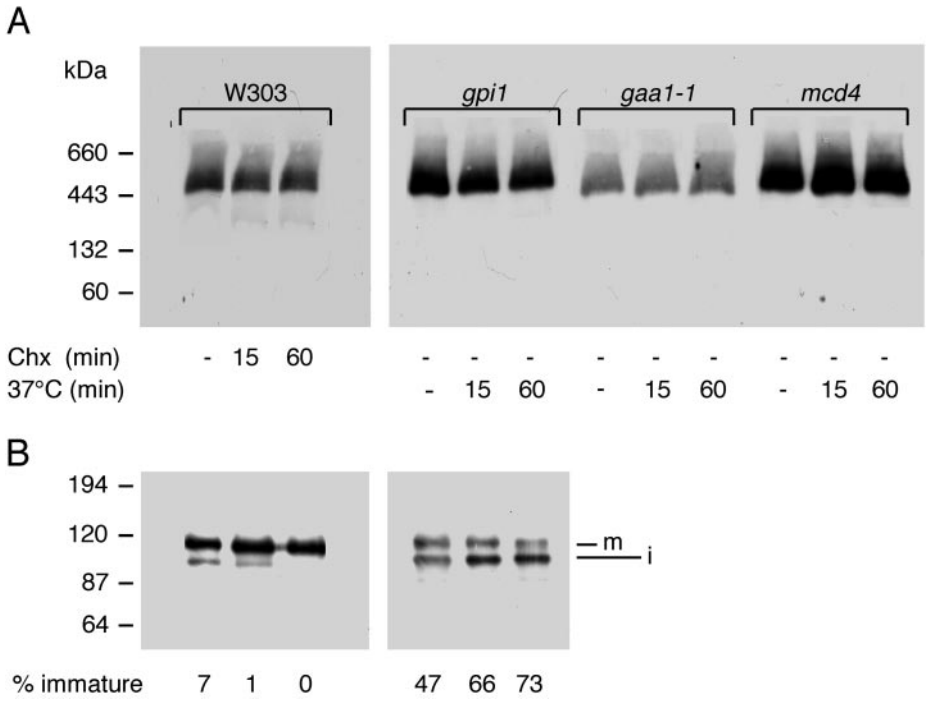


Figure 11. Stability of the transamidase complex in the absence of GPI protein and GPI lipid precursors. (A) W303 cells were grown to exponential phase at 37°C in YPD medium. Cycloheximide (Chx; 100 μg/ml) was added 0, 15, or 60 min before protein extraction. Thermosensitive mutant cells *gpi1, gaa1–1,* and *mcd4* were grown to exponential phase at 24°C in YPD medium. The cells were left at 24°C (-) or were shifted to 37°C for 15 or 60 min. Then, microsomal membranes were prepared and proteins were processed for blue native PAGE. Five μg per lane of solubilized proteins was loaded on a 5–15% gradient gel. Proteins were transferred onto a PVDF membrane and were probed with antibodies against Gpi8p. (B) Five micrograms of the same proteins from W303 and *gpi1* were separated on SDS-PAGE (7.5%). Mature (m) and immature (i) forms of Gas1p were detected by Western blotting with the use of rabbit anti-Gas1p antibodies. The percentage of immature Gas1p, as quantitated by densitometry, is indicated below. Similarly to the original *gpi1* isolate and to *gpi1*Δ cells (Leidich *et al.*, 1994; Leidich and Orlean, 1996), the *gpi1* mutant used here neither grows nor incorporates [2-^{3}H]-*myo*-inositol into proteins at 37°C, whereas at 24°C GPI anchoring is only partially deficient. This partial deficiency explains the abnormally high percentage of immature Gas1p in *gpi1* already at 24°C.

tion and blue native gel electrophoresis of all three complexes since complexes of the same size are solubilized by digitonin alone. However, it is evident that the addition of 6-aminocaproic acid can increase the efficiency of solubilization, especially for the Sec61 complex.

The GPI High-Molecular-Weight Complex Persists in the Absence of GPI Lipids and Precursor Proteins

Although a stable interaction of Gpi8p and Gaa1p has been demonstrated by coimmunoprecipitation (Ohishi *et al.*, 2000), it appeared possible that proteins in the GPI transamidase would not remain firmly associated throughout the catalytic cycle but that the normal workings of this complex required that some subunits dissociate at a certain stage. In fact, if overexpression is used to demonstrate the interaction, a large proportion of overexpressed proteins may never be engaged in GPI anchoring, and this could lead to the artificial perpetuation of a normally transient interaction. Blue native gel electrophoresis allowed detection of the transamidase complex also in normal wt cells (Figure 11A, lane 1). The following experiments were designed to probe the stability of this physiological transamidase complex under conditions where its substrates are depleted. As shown in Figure 11, Gpi8p remained completely confined within the complexes of 430–650 kDa when prepro forms of GPI proteins were depleted with the use of cycloheximide, a treatment that was effective since it led to the complete disappearance of the immature 105-kDa form of the GPI protein Gas1p (Figure 11B). The transamidase complex also persisted after a temperature shift of mutants that, upon a shift to 37°C, block the biosynthesis of GPI lipids at very early stages (*gpi1* and *mcd4*) or interrupt the transfer of GPI lipids onto proteins (*gaa1*) (Hamburger *et al.*, 1995; Leidich and Orlean, 1996; Gaynor *et al.*, 1999; Packeiser *et al.*, 1999). For *gpi1*, the efficiency of the block was assessed by following the gradual accumulation of the immature Gas1p (Figure 2B). In an independent experiment, we more carefully quantitated the amount of Gpi8p in the extracts shown in Figure 11 by SDS-PAGE/Western blotting, whereby the amount of protein loaded onto gels was verified by Coomassie blue staining of gels and blots (not shown). This demonstrated that the Gpi8p content of cells remains constant during 1 h of substrate deprivation, as was done here. Moreover, all cells contain similar amounts of Gpi8p. In Figure 11A, less Gpi8p is detected in *gaa1* simply because less protein was loaded onto the gel in this mutant.

DISCUSSION

Biosynthesis of secretory proteins requires processing events such as insertion into the ER, removal of the signal peptide, and N-glycosylation, events that are operated by heteromultimeric complexes. The existence of a similar complex for the GPI anchor addition has been postulated before based on genetic experiments showing that the overexpression of active-site mutant alleles of Gpi8p such as the C199A allele blocks GPI addition by the wt Gpi8p that is also present in the same cell (Meyer *et al.*, 2000). This dominant-negative effect could be overcome by the concomitant overexpression of wt Gpi8p, suggesting that the C199A and wt forms of Gpi8p are competing for other subunits that are required for activity (Meyer *et al.*,

2000). On the other hand, the dominant-negative effect was not alleviated by the overexpression of Gaa1p, suggesting that other protein components beyond Gaa1p may be required for the formation of a functional complex (Meyer *et al.*, 2000).

Here we indeed isolate a further component, Gpi16p, which is stably associated with Gaa1p and Gpi8p. The functional consequences of its depletion suggest that *GPI16* is essential for yeast because it is required for GPI anchoring of proteins. Under depletion the complete precursor CP2 accumulates. This demonstrates unequivocally that Gpi16p is not required for the biosynthesis of GPI lipids but rather for their attachment to GPI proteins.

Although the functional importance of Gpi16p, as well as that of Gaa1p and Gpi8p, for the attachment of GPI lipids to newly made GPI proteins in the ER is beyond doubt, much remains to be learned about the exact function of these proteins. The homology of Gpi8p with the C13 thiol protease family suggests that Gpi8p is the subunit that proteolytically cleaves the proform of GPI proteins at the ω site. The other two subunits do not contain any known sequence motif that could give a hint with regard to their particular function. Also, Gaa1p, Gpi8p, and Gpi16p do not share any obvious sequence motif among each other. Gaa1p and Gpi16p may be required for binding of the protein and lipid substrates. As long as the membrane orientation of the GPI biosynthetic pathway is not established beyond doubt, it also remains possible, that they are involved in flipping complete GPI lipids from the cytosolic to the lumenal face of the ER. However, the recently identified ER membrane proteins PIG-B (Gpi10p) and PIG-M, which are involved in earlier steps of GPI biosynthesis, have their likely catalytic residues on the lumenal side of the ER membrane, suggesting that later steps of GPI biosynthesis occur on the lumenal side of the ER and that there is no need to flip complete precursors (Takahashi *et al.*, 1996; Maeda *et al.*, 2001). Finally, it also seems to be conceivable that some subunit would operate ER remodeling steps, by which the primary lipid moiety of GPI proteins is exchanged or modified. Indeed, soon after the addition to proteins all GPI anchors get remodeled in the ER, either in the sense that a C26 replaces a C16 fatty acid on the diacylglycerol type lipid moiety or in the sense that the primary diacylglycerol moiety is replaced by a ceramide (Conzelmann *et al.*, 1992; Sipos *et al.*, 1997).

Blue native gel electrophoresis allows a rapid assessment of the state of the GPI transamidase complex under various conditions. It was used to demonstrate that the digitonin-soluble transamidase complex can have various sizes (430–650 kDa) and that the complex remains intact during affinity chromatography (Figure 2A). The subunits that we identify in the complex are Gpi8p (50 kDa), Gaa1p (70 kDa), and Gpi16p (79 kDa), and together they add up to ~200 kDa. It is possible that the 430–650-kDa complex contains more then one copy of some of these three proteins and/or contains yet other nonidentified subunits that were not stained by silver nitrate (Figure 2B). Blue native gel electrophoresis also shows that the complex is present under conditions in which proteins are expressed at physiological levels. To us, it seemed important to show that the complex is present under physiological conditions. A previous study demonstrated by coimmunoprecipitation that hGpi8p and hGaa1p interact stably when they are strongly overexpressed (Ohishi *et al.*, 2000). In the situation of overexpression, the ER folding machinery may get overwhelmed, and incompletely folded proteins may accumulate and aggregate in a nonspecific way through hydrophobic interactions. (Microsomal ALDH, which was used as a specificity control, does not really rule out this possibility since it is mainly cytosolic and exposes only four amino acids on the lumenal side of the ER (Masaki *et al.*, 1994), whereas the major extramembranous parts of Gaa1p and Gpi8p are lumenal.) However, our data clearly indicate that the complex exists under physiological conditions, not only because of data obtained by blue native gel electrophoresis, but also because the complex could be purified from cells, in which its constitutent proteins were under the control of their physiological promoters: *GPI16* and *GAA1* were transcribed from their normal genomic gene; *GPI8* was deleted but present on a centromeric vector as a GST-tagged recombinant protein. Furthermore, blue native gel electrophoresis showed that Gpi8p is stabilized by the incorporation into the transamidase complex (Figure 1).

Previous experiments have suggested that the transamidase complex is not completely static in as much as the induction of dominant-negative alleles for 1 h completely blocked the addition of GPIs to proteins, whereas the half-life of Gpi8p amounts to 9 h. This suggested that newly made dominant-negative Gpi8p alleles get access to the preexisting complexes, implying that this complex is dynamic (Meyer *et al.*, 2000). Also, soluble forms of Gpi8p could be added to ER membranes stripped of their endogenous Gpi8p and reconstitute transamidase activity (Sharma *et al.*, 2000). Our present experiments (Figure 11) suggest that the complex is relatively stable even in the absence of GPI lipids or GPI precursor proteins, although it still remains possible that the depletion of these substrates was incomplete and that the complete absence of substrate would dissociate the complex. Indeed, the complex was stable not only during digitonin extraction and blue native gel electrophoresis, but also during the purification of the Gaa1p/GST-Gpi8p/Gpi16p complex. But even if we accept that the complex is stable in the absence of substrates, this does not exclude the possibility that the complex partially or totally disassembles each time it has to release products. If such were the case, our data support the view that the subunits can reassemble without previous binding of substrates. Clearly, further studies are required to solve this issue.

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II.2. Functional and Structural Analysis of Yeast Gpi8p, an Essential Component of the Yeast GPI Transamidase Complex.

II.2.1. Experimental procedures

Strains, Growth Conditions and Materials

Escherichia coli strains used for protein expression or DNA propagation were BL21(DE3)(from NOVAGEN, Madison, WI), RY3041, RY3080 (4), and HB101. Yeast strains were *Saccharomyces cerevisiae* W303-1B (*MATα ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3- trp1-1 his3-11,15*), FBY 525 (*MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 lys*$^-$Δ*gpi8::kanMX2*/YEp*GPI8*).

Bacteria were grown on LB medium and yeast strains were cultured as described (1). SDaa medium is SD medium containing 20-60mg/L of each of the 20 amino acids, SGaa is the same but with galactose instead of glucose. Affinity purified antibodies against the His_6-tag were obtained from CLONTECH, Palo Alto, CA. ALTA Bioscience, University of Birmingham, UK and Analytical Research and Services, University of Bern, Switzerland sequenced peptides for us. Oligonucleotide synthesis and DNA sequencing services were provided by MICROSYNTH, Balgach, Switzerland.

Construction of multicopy and single copy vectors harbouring various alleles of GPI8.

YEplac112PF1*:* The promoter (P) region of *S. cerevisiae GPI8* was amplified from YEp*GPI8* (12) by PCR using a forward primer which introduces a SalI site at the 5' end (5'-TATTATATTTGTCGACGGTACCCGGGTATC-3') and a reverse primer introducing a NotI and a NdeI site at the 3' end (5'-TATTATATTTGCGGCCGCATATGTTTGTTCG GCTTTTG-3') (restriction sites are underlined). The transcription termination (TT) region of *GPI8* was amplified by PCR using a forward primer which introduces a NotI, NcoI and BglII site at the 5' end (5'-AACTATCACTGCGGCCGCCATGGAGATCTTAAAACAC

TTTTATCC-3') and a reverse primer introducing a SacI site (5'-TATTATATTTGAGCTCTCTAGAGGATCAG-3') at the 3' end. These two PCR fragments were digested with SalI/NotI (P region) and NotI/SacI (TT region) and inserted into the multiple cloning site of the YEplac112 multicopy vector opened with SalI and SacI, thus yielding the plasmid YEplac112PF1. This plasmid contains the P and the TT regions of the *S. cerevisiae GPI8* flanking a multiple cloning site (NdeI-NotI-NcoI-BglII). Introduction of *GPI8* mutant alleles into YEplac112PF1 and YCplac22 : Truncated *GPI8* alleles were generated by PCR using the primers listed in Table I. These primers introduce a NdeI site at the 5' site of the amplified fragment and a BglII site at its 3' end. The PCR fragments were digested with NdeI and BglII and introduced into YEplac112PF1, thus yielding YEplac112PF1-*GPI8* vectors. Fragments comprising P*GPI8-gpi8*-TT*GPI8* were subsequently excised from these vectors as a SalI/SacI fragment and inserted into the centromeric YCplac22 vector digested with SalI and SacI, thus yielding the YCplac22-*GPI8* vectors.

Table I. **Primers used for generation of *GPI8* fragments**

Start	**forward primers**[b]
1[a]	5'-TCGAGTTGCA**CATATG**CGTATAGCAG-3'
23	5'-TCGAGTTGCA**CATATG**AACACAGATGCT-3'
Ending	**reverse primers**
411	5'-ACGTTGAGCT**AGATCT**TCAATTAGTGTACAG-3'
379	5'-ACGTTGAGCT**AGATCT**TCATTTGAAGGAGGT-3'
373	5'-ACGTTGAGCT**AGATCT**TCAACATTCGTTGGA-3'
372	5'-ACGTTGAGCT**AGATCT**TCATTCGTTGGAATC-3'
361	5'-ACGTTGAGCT**AGATCT**TCAAGTAGACTTGAT-3'
347	5'-ACGTTGAGCT**AGATCT**TCAAATCTTGGAGGA-3'
316	5'-ACGTTGAGCT**AGATCT**TCAATGACTTACGGA-3'
291	5'-ACGTTGAGCT**AGATCT**TCATTCTGACGGATT-3'

[a] The numbers at the left indicate the residue in *GPI8* at which a fragment starts or ends, when the corresponding primer is used for PCR. [b] The restriction sites are in bold and regions homologous to *GPI8* are underlined.

Expression of soluble recombinant Gpi8p in bacteria.

For the construction of the expression vector pET15b-*GPI8* the DNA corresponding to amino acids 23 to 379 of yeast *GPI8* was amplified by PCR using a forward primer which introduces a NdeI site at the 5' end of the gene (5'-TCGAGTTGCACATATGAACACAG ATGCT-3') and a reverse primer containing a BamHI site (5'-ACGTTGAGCTGGATCCT CATTTGAAGGAGGT-3'). The fragment was inserted into the T7 RNA polymerase-based expression vector pET15b (NOVAGEN). The resulting pET15b-*GPI8* produces a soluble Gpi8p-fragment with a 20-amino acid N-terminal extension containing a His_6-tag plus a thrombin cleavage site allowing to remove the His_6-tag. For the construction of the expression vector pET22b-*GPI8* the DNA corresponding to amino acids 23 to 379 of yeast *GPI8* was amplified by PCR using a forward primer which introduces a NdeI site at the 5' end of the gene (5'-TCGAGTTGCACATATGAACACAGATGCT-3') and a reverse primer coding for a thrombin cleavage site (LVPRGS, italic) and containing a XhoI site (5'-ACGTTGAGCTCTCGAG*GCTGCCGCGCGGCACCAG*TTTGAAGGAGGT-3'). The fragment was inserted into the T7 RNA polymerase-based expression vector pET22b (NOVAGEN). The resulting pET22b-*GPI8* produces a soluble Gpi8p fragment with a 15-amino acid C-terminal extension containing a thrombin cleavage site and a His_6-tag. pET15b-*GPI8* and pET22b-*GPI8* were used to transfect *E.coli* strains BL21(DE3), RY3041 and RY3080. The correct insertion of all fragments into vectors was verified by DNA sequencing.

Purification of soluble Gpi8p

Preparation of soluble extracts. BL21(DE3) bacteria transfected with pET15b-*GPI8* or pET22b-*GPI8* were grown at 16°C until they reached an OD_{600} of 0.6 to 1.0 whereupon the expression of the recombinant protein was induced by addition of isopropylthiogalactoside (IPTG) to 0.8mM. After 24 h of culture, the same amount of IPTG was added once more. After 48h, cells were harvested and resuspended in 50ml of buffer 1 (B1, 50mM Tris-HCl pH 7.9, 150mM NaCl, 2.5mM β-mercaptoethanol, 1mM of PMSF). The cells were lysed by snap freezing in liquid nitrogen, thawed and sonicated by a probe sonicator for 5min at 4°C. The extract was clarified by centrifugation for 1h at 40'000 rpm (37'000*g)* at 4°C in a Sorvall Ti 1250 fixed angle rotor.

Purification of soluble Gpi8p: The soluble extract (50ml, corresponding to 1 liter of *E.coli* culture) was loaded on a 4ml metal-chelating column (TALON Superflow, CLONTECH, 5x200mm, preequilibrated in B1) at 4°C. The column was washed with B1 and then with B1 containing 10mM imidazole; the adsorbed proteins were eluted with a linear gradient (30ml) going from 10 to 250mM imidazole in B1. The fractions containing Gpi8p$^{23-379}$-His_6 (as judged by SDS-PAGE) were pooled and concentrated using a CENTRIPREP (AMICON, Beverly, MA) device with a cut-off at 3'000 Da.

For further purification the protein was loaded onto a Superose 12 gel filtration column (10x300mm, fractionation range: 10^3-$3x10^5$ Da, AMERSHAM PHARMACIA Biotech, Buckinghamshire, UK) equilibrated in buffer 2 (B2; 20mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM β-mercaptoethanol). The column was eluted with B2 at a flow rate of 0.5ml/min at 4°C. To remove the His_6-tag, we used buffer 3 (20mM Tris-HCl, pH 7.9, 2.5mM β-mercaptoethanol) and concentrated by ultrafiltration using a CENTRIPREP device to a final concentration of 1-2 mg/ml. The concentrated protein, adjusted to a final concentration of 2.5mM $CaCl_2$, was then incubated overnight with thrombin (SIGMA, St.Louis, MO; 1.0 unit/mg of Gpi8p-His_6) at 4°C . After filtration (Supor Acrodisc 0.2μm, GELMAN SCIENCES , Ann Arbor, MI) the sample was loaded at a flow rate of 0.5ml/min onto a MonoQ column (AMERSHAM PHARMACIA, 5x50mm) equilibrated in buffer 4 (B4; 20mM Tris-HCl pH 7.9, 50mM NaCl, 2.5mM β-mercaptoethanol). The column was washed with the same buffer and elution was performed with a linear NaCl gradient (20ml) from 50 to 750mM NaCl in B4. Gpi8p-containing fractions were pooled and reloaded onto the metal-chelating column in order to eliminate the SlyD contaminant. The flow-through was recovered, concentrated to 2mg/ml and either used immediately for activity studies or dialyzed against 10mM Tris-HCl, 250mM NaCl, 2.5mM β-mercaptoethanol, 10% glycerol and kept at – 80°C.

In vitro protease assays using rhodamine labeled peptides

Rhodamine labeled peptides containing the ω-site of the GPI protein YOR009w (Rhodamine-IVEQTE**N**GAAKAV-COO^-, ω-site in bold) was used as substrate in order to try to detect proteolytic activity of purified Gpi8p$^{23-379}$. As one standard we used the peptide G6 (Rhodamine-IVEQTE**N**-COO^-) corresponding to the product we expect if the

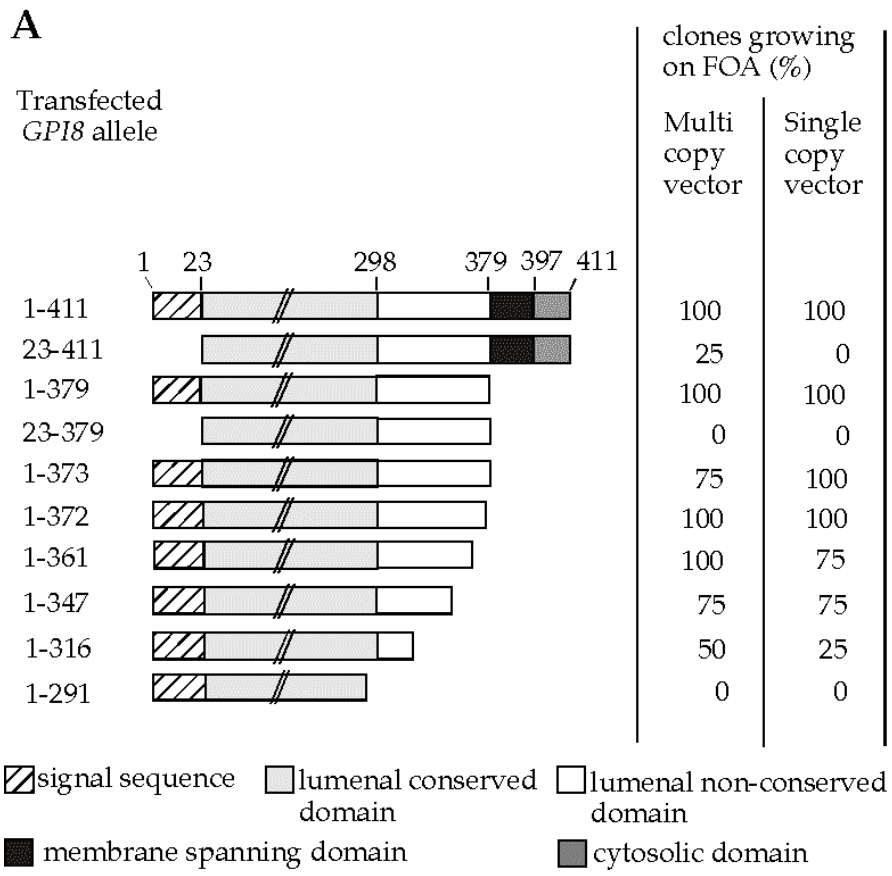
YOR009w is cleaved at its ω site. Peptides A1 (Dye-LRRASLG-COO^-) and C1 (Dye-PLSRTLSVAAK-COO^-) from the PepTag protease assay (PROMEGA, Madison, WI) were used for detecting contaminating proteases. Any digestion altering the size and charge of the peptides was detected using agarose gel electrophoresis. In the standard assay the YOR009w peptide (2μg) was incubated in presence of variable amounts (0-20μg) of Gpi8p$^{23-379}$-His_6 or Gpi8p$^{23-379}$ (His_6-tag removed) in various reaction buffers (50mM Tris-HCl pH 8.0, 50mM HEPES pH 7.0, 50mM MES pH 6.0, +/- 5mM $CaCl_2$, +/- 5mM $MgCl_2$, +/- 10mM hydrazine, +/- 10mM hydroxylamine, +/- ethanolamine, +/- mannose, +/- *myo*-inositol). Different incubation times (0-24hours) and incubation temperatures (20-40°C) were used. At the end of the incubation, 1μl of 80% glycerol was added, and samples were loaded into the wells of a 1% agarose gel, equilibrated in 50mM Tris-HCl pH 8.0 and electrophoresed at 44 volts until the separation of the various standard peptides was apparent.

II.2.2. Results

II.2.2.1. The membrane spanning domain of Gpi8p is dispensable for activity.

In order to purify yeast Gpi8p for crystallization we decided to investigate whether its hydrophobic MSD is required for activity. Indeed this MSD is entirely lacking in *C. elegans* and some protozoan Gpi8p. The full length (1-411) or truncated versions of yeast Gpi8p under the control of their natural promoter were inserted into centromeric or multicopy vectors as depicted in Fig. 1A and their functionality was tested in a *Δgpi8* strain kept alive by the *URA3*-based YEp*GPI8* plasmid. All clones which were obtained after plasmid shuffling (Fig. 1A) grew at a normal rate except for the ones harbouring Gpi8p$^{1-316}$, which only grew slowly. The results allow the following conclusions: 1) The cytosolic and the MSD of yeast Gpi8p are dispensable for function. 2) The hydrophilic domain preceding the MSD can be removed down to amino acid 316 with only partial loss of activity. 3) Truncation into the conserved domain (D2 in Fig. 1C) completely destroys the activity. These data are largely identical with what was recently reported for human Gpi8p (3).

To formally demonstrate that Gpi8p has to reside in the lumen of the ER in order to be functional, we also tested a construct lacking amino acids 1-22. When expressed from a high copy number vector, it was able to rescue *Δgpi8* cells. Simultaneous removal of the C-terminal MSD rendered the construct non-functional. Thus, it appears that the N-terminal signal sequence is not required for transamidase activity and that the C-terminal MSD can direct membrane insertion of Gpi8p in the correct orientation at a low rate (see below). The result also suggests that Gpi8p cannot function if it is in the cytoplasm.To see if the truncated forms of Gpi8p allowing for a normal growth rate were expressed at physiological levels, we quantitated Gpi8p on Western blots. Normal cells contain 46, 48 and 50 kDa forms of Gpi8p carrying 1, 2 and 3 N-glycans, respectively (1). In agreement with previous results (2), *Δgpi8* cells transfected with full length*GPI8* on a YEp vector contained about 4 fold more Gpi8p than wild type (wt) cells (Fig. 2A, lanes 1, 2). As shown in Fig. 2, also other constructs introduced on YEp vectors (lanes 2,3,5,7,9) were expressed at significantly higher than wt levels whereas constructs introduced on centromeric vectors (YCp)(lanes 4,6,8,10) were expressed at about physiological levels.



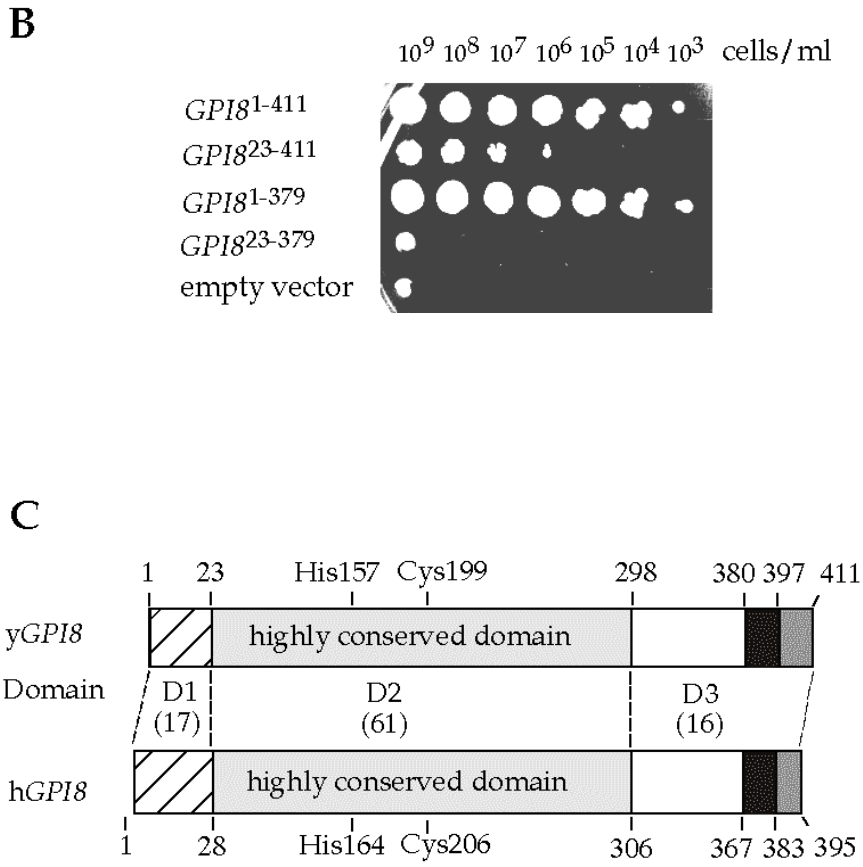


FIG. 1. ***In vivo*** **analysis of *GPI8* mutants** A, the haploid strain FBY525 (*Δgpi8*/YEp*GPI8*) was transfected with multi- (YEplac112PF1) or the single copy (YCplac22) vectors harbouring truncated alleles of *GPI8* under the control of the *GPI8* promoter. 8 clones from each transfected strain were streaked out on SDaaUA plates containing FOA to see if the wt *GPI8* (on YEp*GPI8-URA3*) could be forced out. The percentage of clones giving viable progeny on FOA is indicated. B, FBY525 (*Δgpi8*/YEp*GPI8*) transfected with YEplac112PF1 harbouring various truncated alleles of *GPI8* were spotted with a frogging device onto SDaaUA/FOA plates, and were incubated for 3 days at 30°C. Concentrations (cells/ml) used for frogging are shown at the top. C, schematic representation of yeast and human Gpi8p (yGpi8p/hGpi8p divided into domains D1-D3. Positions of domain boundaries and the active site His and Cys residues are indicated.

Removal of the N-terminal signal sequence (amino acids 1-22)(lane 4) leads to the appearance of a major band of 43.5 kDa that is close to the theoretical mass of Gpi8p^{23-}

^{411} (45.07 kDa) and most probably represents cytosolic Gpi8p$^{23\text{-}411}$ but generates also minor bands at 46 and 48 kDa having the molecular weight we expect if Gpi8p$^{23\text{-}411}$ were translocated and N-glycosylated on one or two sites. (Removal of amino acids 1-22 actually destroys the third N-glycosylation site, N^{23}, since N-terminal asparagins are not substrates for the oligosaccharyltransferase.) It must be these minor 46 and 48 kDa forms which are functional.

It is interesting to note that C-terminally truncated Gpi8p alleles are significantly overexpressed in comparison with other constructs or wt *GPI8* introduced on the same YEp vector (lanes 2, 5, 7, 9). The cells may attempt to increase their GPI anchoring capacity by overexpressing these alleles, especially in the case of Gpi8p$^{1\text{-}316}$, which evidently is less functional than the other constructs (Fig. 1A). This may be achieved through some regulatory phenomenon or there may be selection for cells that have high copy numbers of the YEp plasmids during culture. Interestingly, the overexpression of Gpi8p alleles lacking the C-terminal MSD is associated with the appearance of a smear in the high molecular weight range, most likely due to elongation of N-glycans in the Golgi (Fig. 2, lanes 5, 7, 9). This smear is not observed on very long exposures of the lanes containing the same alleles expressed from a centromeric vector (not shown). Overexpression of these alleles also leads to the appearance of lower molecular weight forms, which may represent breakdown products, possibly generated in the vacuole. Thus, the mechanism retaining C-terminally truncated Gpi8p alleles in the ER, whatever its mode of action, seems to be saturable or leaky. From this we can conclude that the MSD of wt Gpi8p can serve to retain Gpi8p in the ER, a property which only becomes important if it is overexpressed. The truncated Gpi8p forms reaching the Golgi may be transported to the vacuole but they apparently are not secreted into the culture medium, since we could not detect any Gpi8p in Western blots of the culture media of the cells analyzed in Fig. 2 (not shown). The truncated Gpi8p forms reaching the vacuole are probably processed as we can see it in Fig.3, lanes 6 and 7 obtained after treating the proteins with endoglycosidaseH. In this case, the overexpression of Gpi8p allele lacking the C-terminal MSD, associated with the appearance of a smear (Fig.2, lane 7, Fig.3, lane 6), is also associated with the appearance, in excess, of a new fragment (Fig.3, arrow c, 34kDa), in comparison with the expression, in physiological levels, of the same Gpi8p allele (Fig.3, arrow b, 38kDa).

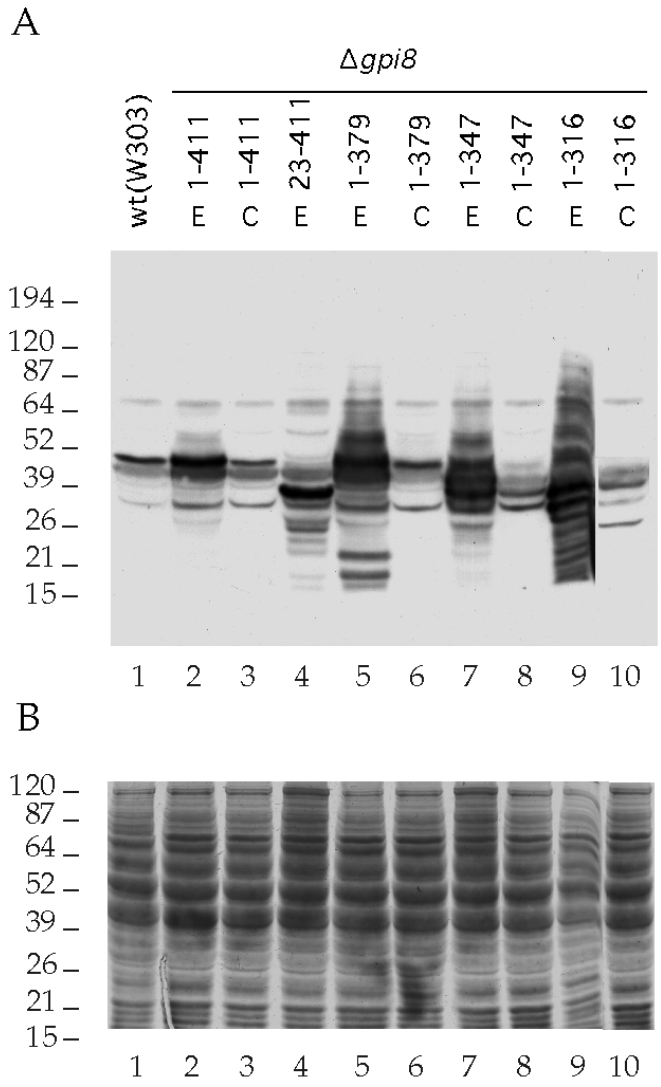


FIG. 2. **Detection of truncated Gpi8p forms by Western blotting** Wild type (W303-1B) and *Δgpi8* cells transfected with an episomal (E) or a centromeric (C) vector harbouring various truncated alleles of *GPI8* were inoculated at 0.1 OD_{600} and cultured overnight at 24°C. The different cells grew to 1.4 –1.8 OD_{600}. Proteins were extracted by incubating cells for 5 min in NaOH and boiling at 95°C in reducing sample buffer (8). Samples were run on a 12% SDS-PAGE and probed with rabbit anti-Gpi8p (1). Each lane contains the equivalent of 2 OD_{600} units of cells. B, The gel remaining after protein transfer was stained with Coomassie blue to verify that equivalent amounts of protein had been extracted from all cells. The blot was also stained with Coomassie blue and showed the same image with only lane 9 having slightly less protein than the other lanes (not shown).

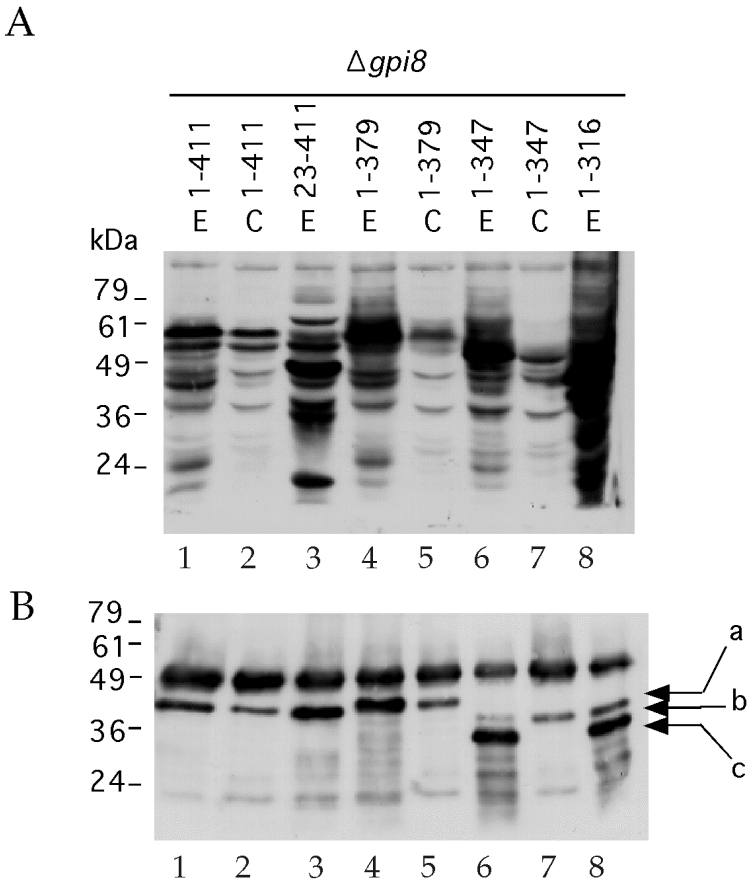


FIG. 3. **Analysis of the truncated Gpi8p forms by EndoglycosidaseH treatement.** A. *Δgpi8* cells transfected with an episomal (E) or a centromeric (C) vector harbouring various truncated alleles of *GPI8* were inoculated at 0.1 OD_{600} and cultured overnight at 24°C. The different cells grew to 1.4 –1.8 OD_{600}. Proteins were extracted by incubating cells for 5 min in NaOH and boiling at 95°C in reducing sample buffer (8). Samples were run on a 12% SDS-PAGE and probed with rabbit anti-Gpi8p (1). Each lane contains the equivalent of 2 OD_{600} units of cells. B. proteins from the corresponding cells were extracted, immunoprecipitated with anti-Gpi8p antibodies, and treated with endoglycosidaseH for 24h at 30°C. Proteins were then precipitated with TCA, run on a 12% SDS-PAGE, and probed with rabbit anti-Gpi8p. The band observed at the top of the figure, and present in homogenous levels in each lane, corresponds to the heavy chain of the antibody used for the immunoprecipitation of Gpi8p.

II.2.2.2. Purification of Gpi8p$^{23\text{-}379}$

A His_6-tag was placed at either the N- or the C-terminal end of the soluble 23-379 fragment, which according to the above studies is fully functional. The 23-379 fragment was expressed as a cytosolic protein in *E. coli* and was purified by affinity chromatography on a metal chelating column. When the His_6-tag was placed at the N-terminus, a major 45 kDa band corresponding to the expected size plus several minor bands were obtained (Fig. 4B, lane N). Minor bands suggested either premature termination of transcription or translation or proteolytic degradation at the C-terminal end. In contrast, no heterogeneity of affinity purified products was seen when the His_6-tag was placed at the C-terminus (Fig. 4B, lanes C). Fractions 6 to 13 eluting at 25 to 65mM imidazole contained the most of the desired protein. They were however contaminated by a 24 kDa protein (Fig. 4C), which could neither be removed by anion exchange chromatography on MonoQ nor by gel filtration on Superose12 columns (Fig. 4E). Protein sequencing of the 24 kDa protein yielded the sequence KVAKD, a sequence corresponding to the N-terminal end of SlyD, a protein of *E. coli* (SWISSPROT: P30856). SlyD contains several His-rich stretches and has been observed to be retained by metal chelating columns before (4). SlyD could not easily be resolved from $Gpi8p^{23-379}$-His_6, since it has a similar mass and pI as $Gpi8p^{23-379}$-His_6. Indeed, SlyD forms a dimer of 41.7 kDa and has a theoretical pI of 4.86 whereas $Gpi8p^{23-379}$-His_6 has a molecular mass of 43.1 kDa and a theoretical pI of 4.81. SlyD is induced by IPTG only in cells harbouring the pET22b-*GPI8* vector but not in non-transfected cells (Fig. 4C). SlyD is a member of the FKBP family of cis-trans peptidyl-prolyl isomerases and thus seems to be induced by the cytosolic accumulation of $Gpi8p^{23-379}$-His_6. To get rid of this contaminant we tried to express $Gpi8p^{23-379}$-His_6 in SlyD mutant strains of *E. coli*. As can be seen on Fig.4D, $Gpi8p^{23-379}$-His_6 gets partially degraded in such strains, potentially because, without the aid of SlyD, the protein fails to achieve its normal tridimensional structure. The SlyD contaminant could however be eliminated in spite of its physicochemical similarity to $Gpi8p^{23-379}$-His_6: After removal of the C-terminal His_6 extension by thrombin, $Gpi8p^{23-379}$ ran straight through the TALON column whereas SlyD and residual, uncleaved $Gpi8p^{23-379}$-His_6 was still retained. This procedure yielded pure $Gpi8p^{23-379}$ as shown in Fig. 4E, lane 6.

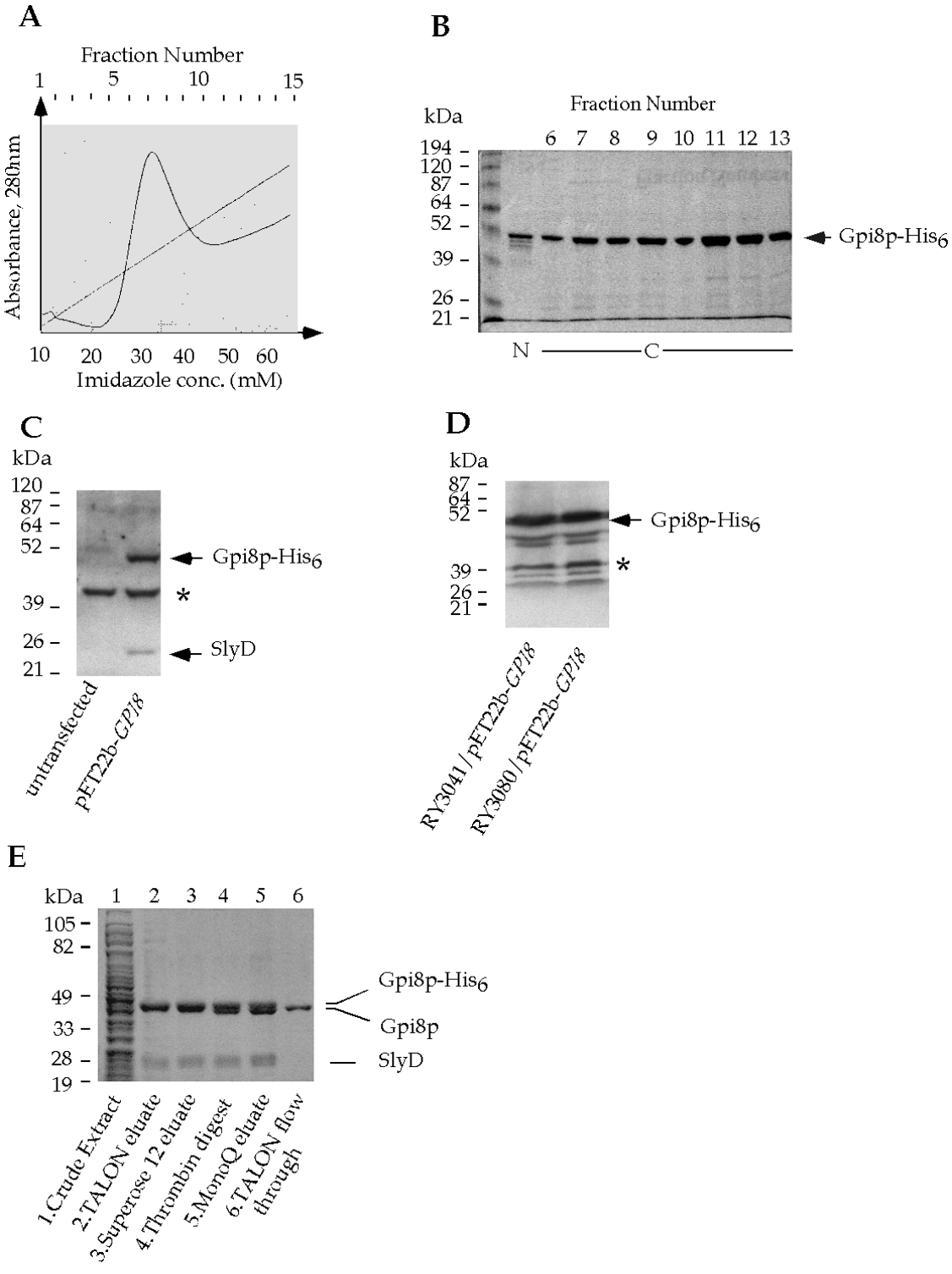


FIG. 4. **Purification of Gpi8p$^{23\text{-}379}$ from *E. coli*.** Gpi8p$^{23\text{-}379}$-His$_6$ was purified from induced *E. coli* (BL21(DE3)) harbouring either pETb15-*GPI8* (B, lane N) or pET22b-*GPI8* coding for constructs with the His$_6$-tag on the N- and C-terminus of *GPI8*$^{23\text{-}379}$,

respectively. A, Extract from cells containing pETb22-*GPI8* were passed over an immobilized metal affinity chromatography sorbant (TALON) using a FPLC system and eluted by a gradient of imidazole. Protein concentration in the eluate was monitored by the absorbance at 280nm. B, fractions obtained from TALON columns were analyzed on a 12% polyacrylamide gel (w/v), the resulting gel was stained with Coomassie Blue to detect proteins. Lane N contains Gpi8p$^{23\text{-}379}$-His$_6$ from cells containing pET15b-*GPI8* (His$_6$-tag on N-terminus), lanes C from cells containing pET22b-*GPI8* (His$_6$-tag on C-terminus). C, untransfected or pET22b-*GPI8* transfected BL21(DE3) were induced by IPTG, crude extracts were prepared and analyzed by SDS-PAGE followed by Western blotting using affinity purified anti-His$_6$ antibodies. (*) indicates a band that is detected by anti-His$_6$ antibodies but is not retained on TALON columns. D, pET22b-*GPI8* was transfected into ΔSlyD strains (RY3041 and RY3080), Gpi8p-His$_6$ was induced, cell extracts were subjected to SDS-PAGE and Western blotting with anti-His$_6$ antibody. E, aliquots of the crude bacterial extract and of material recovered after five subsequent purification steps were analyzed by SDS-PAGE followed by Coomassie blue staining.

II.2.2.3. Functional analysis of purified Gpi8p$^{23\text{-}379}$-His$_6$

Gpi8p is thought to remove the C-terminal GPI anchoring signal using the classical mechanism of Cys proteases so that the carbonyl of the amino acid in ω position is transiently bound via a thioester linkage to the active site Cys of Gpi8p, allowing the C-terminal anchoring signal to leave. In a second reaction the enzyme would be displaced from the GPI protein substrate by the preformed GPI. Hydrazine and hydroxylamine are nucleophiles, which have been shown to aid the enzymatic removal of the C-terminal GPI anchoring signal in the microsomal system (9). They are thought to mediate this second reaction by attacking the thioester bond between the active site Cys and the GPI protein, thus acting as a substitute for the natural GPI. Having large amounts of purified Gpi8p$^{23\text{-}379}$-His$_6$ we used it to screen for proteolytic activity against a variety of peptides either mimicking the sequence around the ω site of yeast GPI proteins or peptides which are cleaved by a large range of specific and unspecific peptidases and are widely used to detect proteolytic activities. Peptides were incubated with purified Gpi8p$^{23\text{-}379}$-His$_6$ at

different pH, in the presence or absence of hydrazine, hydroxylamine, ethanolamine, mannose, inositol, (to mimic the presence of a GPI), in various combinations and with various concentrations of reducing agents to preserve the active site cysteine. All peptide substrates were completely stable, even in incubations up to 24 h, but Gpi8p failed to cleave them, while crude yeast extracts readily degraded all of them. This negative result may simply be due to the fact that the pure Gpi8p fragment had not attained its correct conformation in bacteria or had lost its activity during purification. On the other hand, it may well be that Gpi8p needs additional components, which activate the protein. One potential activation component is Gaa1p, which also is essential for GPI anchoring and which has recently been shown to interact with Gpi8p (3).

II.2.3. Discussion

Gpi8p forms lacking a C-terminal MSD can functionally replace wt Gpi8p. It has to be taken into consideration that Gpi8p is near to rate limiting for cell growth in wt cells (2). Since C-terminally truncated Gpi8p versions, expressed at about physiological levels support a normal growth rate, we have to assume that these constructs are efficiently retained in the ER although they are swamped out of the ER when they are overexpressed (Fig. 2). Thus, the ER must contain a limited number of binding sites for Gpi8p, which have sufficient affinity to retain the soluble Gpi8p alleles. These sites may well be provided by Gaa1p.

The data in this report also suggest that the MSD may help to retain Gpi8p in the ER, either by stabilizing an interaction with Gaa1p or by a completely unrelated mechanism. This may not be of importance under normal growth conditions but may be advantageous under stress conditions. Thus, the efficient retention of Gpi8p observed in wt cells may be the result of several independently operating factors which are 1) the interaction of its hydrophilic domain with Gaa1p, 2) the retention effect of its MSD and 3) the integration into a big protein complex, which, because of its size, may be less prone to exit the ER than smaller proteins.

It came as a surprise that *GPI8* constructs lacking an N-terminal signal sequence for insertion into the ER were nevertheless able to rescue Δ*gpi8* cells. The C-terminal MSD of

Gpi8p can apparently direct proper membrane insertion of Gpi8p, although not very efficiently. Indeed, analysis of charges of the 15 residues on either side of this hydrophobic sequence shows a difference of 3 charges and predicts that insertion directed by this sequence would position Gpi8p correctly with its N-terminal hydrophilic domain oriented towards the ER lumen (5). It is possible that yeast is more apt for such unconventional membrane insertion than higher organisms because posttranslational membrane insertion into the ER is a relatively common event in yeast while mammalian cells almost exclusively utilize the cotranslational translocation mode (6, 7).

II.2.4. References

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II.3. Structural Organization of the Transamidase Complex : a Two-Hybrid-based Model

The yeast *S.cerevisiae* and the two hybrid systems are essentiel genetic tools for studying the macromolecular interactions that define all living organisms. The two-hybrid system makes use of the observation that transcription factors are generally composed of two separated domains, a sequence-specific DNA-binding domain (DB), and a transactivation domain (AD). When a protein X is fused to DB and a protein Y to AD, an interaction between X and Y tether the DB to the AD, allowing the reconstitution of a functional transcription factor, thus activating a downstream reporter like lacZ(1). A regularly upgrade World Wide Web site summarizes the experiences of many investigators (http://www.fccc.edu:80research/labs/golemis/InteractionTrapInWork.html). In our study, we used the *GAL4*-based two hybrid system to verify the binding of Gpi8p to Gaa1p (performed before we identified the Gpi16p), and to study the macromolecular organization of the GPI-transamidase Complex (performed recently, after we identified the Gpi16p, and after the identification of Gpi17p had been reported (Ohishi *et al*., 2001).

II.3.1. Experimental procedures

Strains, Growth Conditions and Materials

Yeast trains : Y187 (*MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δmet⁻ gal80Δ URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*), PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δgal80Δlys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) containing *HIS3, ADE2* and *lacZ* behind the *GAL1, GAL2* and *GAL7* promoters, respectively (2)

Two hybrid assay for the binding of Gpi8p to Gaa1p

Two hybrid studies required the construction of vectors containing fragments of Gaa1p and Gpi8p fused to the DNA-binding domain (BD) or the transcription activation domain (AD) of the transcription factor Gal4p. To fuse the *GAL4* BD and the *GPI8*$^{23-379}$ fragment, the *GPI8* fragment was amplified by PCR using a forward primer comprising a

NdeI site and a reverse primer containing a BamHI site (primers listed in Table I). The amplified fragment was inserted between the NdeI and BamHI sites of the pAS2.1 vector (CLONTECH) to generate the fusion between the *GAL4* BD and the *GPI8*$^{23\text{-}379}$ fragment. *GAA1* fragments were PCR amplified using forward primers containing a BamHI site and reverse primers containing a XhoI site (Table II) and pDH15 as a template (7). The amplified fragments were ligated into the BamHI and XhoI sites of the pACT2 vector (CLONTECH), thus fusing the fragment to the *GAL4*-AD (Fig. 1B). Vectors containing the *GAL4*-BD fused to *GAA1* fragments and *GAL4*-AD fused to *GPI8*$^{23\text{-}379}$ were similarly prepared (primers listed in tables I and II). Control vectors containing other fusions were: pVA3-1, encoding *GAL4*-BD fused to murine p53 protein in pAS2-1; pTD1-1 encoding *GAL4*-AD fused to SV40 large T-antigen in pACT2; pLAM5-1 encoding *GAL4*-BD fused to human lamin C protein in pAS2-1. The strains Y187 and PJ69-4A were transformed using lithium acetate. β-galactosidase was measured either directly in colonies growing on agar by the Colony Lift Filter assay (3) or in cells grown in liquid culture using ONPG as a substrate as described (4). Antibody against AD of Gal4p was from CLONTECH.

Two hybrid assay for the macromolecular organization of the GPI-Transamidase Complex

The PCR amplified *GPI16*$^{30\text{-}550}$ and *GPI17*$^{30\text{-}470}$ fragments (by using primers listed in tables III and IV) were inserted between the EcoRI and BamHI sites of the pAS2.1 vector to generate the fusions with the *GAL4*-DB and between the BamHI and Xho1 sites of the pACT2 vector to generate the fusions with the *GAL4*-AD.

Table I. **Primers used for generation of *GPI8* fragments**

Start [a]	**forward primers**[b]
23 23	5'-TCGAGTTGCA**CATATG**AACACAGATGCT-3' 5'-TC**GGATCC**AAGCGGCCGCAAACACAGATGCT-3
	reverse primers
379 379	5'-ACGTTGAGCT**GGATCC**TCATTTGAAGGAGGT-3' 5'-ACGT**CTCGAG**AGATCTTCATTTGAAGGAGGT-3'

[a] The numbers at the left indicate the residue at which a fragment starts or ends, when the corresponding primer is used for PCR. [b] The restriction sites are in bold and regions homologous to the corresponding gene are underlined.

Table II. **Primers used for generation of *GAA1* fragments**

Start	**forward primers**
44	5'-TCGAGTTGCA**GGATCC**AAGATGGACAGTAC-3'
50	5'-TCGAGTTGCA**GGATCC**AAAGAAGAACATAC-3'
50	5'-TCG**CATATG**AGGATCCAAAGAAGAACATAC-3'
Ending	**reverse primers**
340	5'-ACGTTGAGCT**CTCGAG**TCAGAACGATTGGTG-3'
350	5'-ACGTTGAGCT**CTCGAG**TCACTGACGTGGTGC-3'
340	5'-ACGTTGAGCT**GGATCC**TCAGAACGATTGGTG-3'

Table III. **Primers used for generation of *GPI16* fragments**

Start	**forward primers**
30	5'-TCGAGTTGCA**GGATCC**AA**GAATTC**AGTTTATGGTATCCGTA-3'
Ending	**reverse primers**
550	5'-TCGAGTTGCA**GGATCC**TTAATACGGCATACTAAAATC -3'
550	5'-TCGAGTTGCA**CTCGAG**CTAATACGGCATACTAAAATC -3'

Table IV. **Primers used for generation of *GPI17* fragments**

Start	**forward primers**
30	5'-TCGAGTTGCA**GGATCC**AA**GAATTC**ACAGTTTATAGAGCAT-3'
Ending	**reverse primers**
470	5'-TCGAGTTGCA**GGATCC**TTATTGTGGGAAGAAATTTTG-3' 5'-TCGAGTTGCA**CTCGAG**CTATTGTGGGAAGAAATTTTG-3'

II.3.2. Results

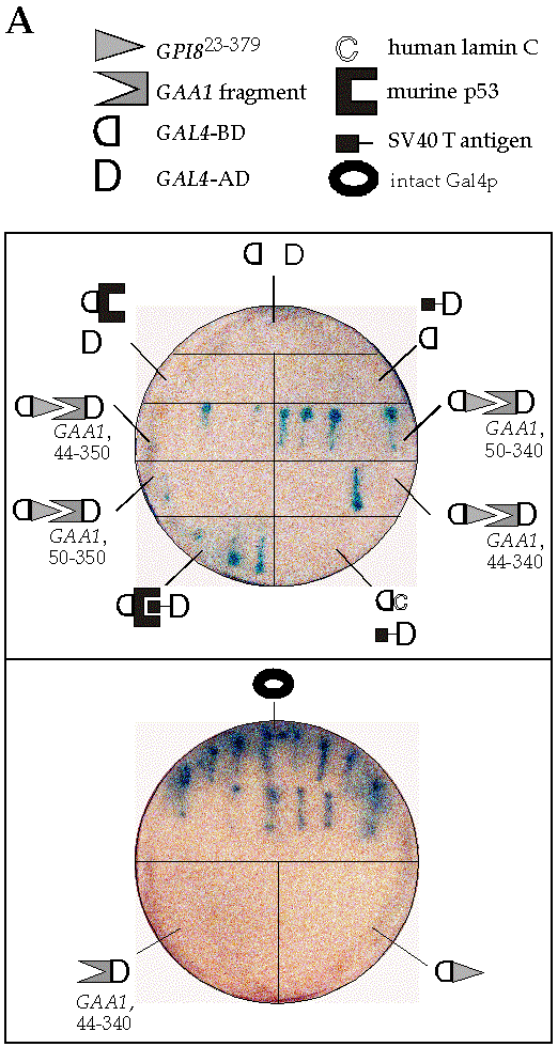
II.3.2.1. The hydrophilic, lumenal parts of Gpi8p and Gaa1p interact directly with each other

Gpi8p$^{23-379}$ was hooked via its N-terminus to the DNA binding domain (BD) of the transcription factor Gal4p. Alternatively it was coupled via its N-terminus to the Gal4p activation domain (AD). Gaa1p contains a lumenally exposed hydrophilic domain, which resides between the single N-terminal and the several C-terminal MSDs. This hydrophilic domain was similarly coupled via its N-terminus to the AD or BD of Gal4p, whereby the fragment boundaries were slightly different in the various constructs. These constructs were transfected either singly or in combination into different tester strains containing reporter genes such as β-galactosidase (lacZ), *HIS3* and *ADE2* behind various galactose inducible yeast promoters capable to interact with the BD of Gal4p. As can be seen in Fig. 1A, several of the clones coexpressing BD-Gpi8p$^{23-379}$ and an AD-Gaa1p construct produced as much β-galactosidase as the positive controls containing either intact Gal4p (Fig. 1A, lower filter) or other interacting partners such as p53 which interacts with SV40 T antigen (Fig. 1A, upper filter). Expression of AD-Gaa1p alone, or of pairs, which do not contain interacting proteins, did not induce detectable β-galactosidase activity. BD-Gpi8p$^{23-379}$ was the only construct able to activate transcription even when expressed alone, albeit at a significantly lower level than in combination with AD-Gaa1p: In the colony lift filter assay, colonies coexpressing BD-Gpi8p$^{23-379}$ and AD-Gaa1p became visibly blue within 50min whereas colonies expressing BD-Gpi8p$^{23-379}$ alone became blue after 210min. When measuring in cells grown in liquid culture, clones coexpressing BD-Gpi8p and AD-Gaa1p reached β-galactosidase levels of 63 Miller units whereas cells expressing either one alone reached 13 and 0 units, respectively. Curiously, not all Gaa1p fragments worked equally well, since the shorter constructs going to amino acid 340 worked better than the longer ones going to amino acid 350 (Fig. 1A). When the fragments of Gpi8p and of Gaa1p were interchanged, i.e. if Gpi8p was attached to the AD, Gaa1p to the BD of Gal4p, the interaction could still be detected, although the signals

were much weaker: Colonies of doubly transfected cells got faintly blue (Y187 transfectants) or started to grow in the absence of His (PJ69-4A transfectants) whereas no signal was obtained in single transfectants (not shown).

The expression levels of the interacting Gpi8p- and Gaa1p-constructs in the strains shown in Fig. 1A were assessed on Western blots using antibodies against a fragment of Gpi8p (Gpi8p$^{100-281}$ (5)) or the AD of Gal4p and densitometry. As shown in Fig. 1B, lane 2, BD-Gpi8p$^{23-379}$ having a theoretical mass of 63.2 can be detected as a 62 kDa band (arrow), which is 11 times less abundant than the endogenous Gpi8p contained in the bands at 46, 48 and 50 kDa. Since BD-Gpi8p$^{23-379}$ by itself already induces a low level of transcription, we feared that the stronger activation of transcription in cells coexpressing BD-Gpi8p$^{23-379}$ may be due to an enhanced expression of BD-Gpi8p$^{23-379}$ in cells coexpressing AD-Gaa1p$^{50-340}$. Comparison of lanes 2 and 3 however shows that this is not the case.

Since no antibodies detecting physiological levels of Gaa1p were available, we compared AD-Gaa1p$^{50-340}$ to AD-Gpi8p$^{23-379}$ (lanes 5, 6), which in turn could be compared to endogenous Gpi8p (lane 8 versus 7). This latter comparison showed that AD-Gaa1p$^{50-340}$ is present at 11.2 fold higher levels than endogenous Gpi8p. Thus, we get a positive signal in the two hybrid assay, if BD-Gpi8p$^{23-379}$ and AD-Gaa1p$^{50-340}$ are present at levels 11 fold lower and higher, respectively, than endogenous Gpi8p. Although we do not know the physiological ratio of Gaa1p to Gpi8p in normal cells these estimations suggest that the observed two hybrid interaction occurs at physiologically relevant concentrations. Furthermore, the two hybrid results argue that the Gpi8p and Gaa1p interact with each other through their water soluble domains, which reside within the lumen of the ER. The same was proposed for the human system (6). Also, the results clearly indicate that no other ER resident protein is required to mediate this interaction. It can however not be excluded that other proteins stabilize or disrupt the interaction of Gpi8p and Gaa1p under physiological conditions.


A
GPI8 23-379
GAA1 fragment
GAL4-BD
GAL4-AD
human lamin C
murine p53
SV40 T antigen
intact Gal4p
GAA1, 44-350
GAA1, 50-350
GAA1, 50-340
GAA1, 44-340
GAA1, 44-340

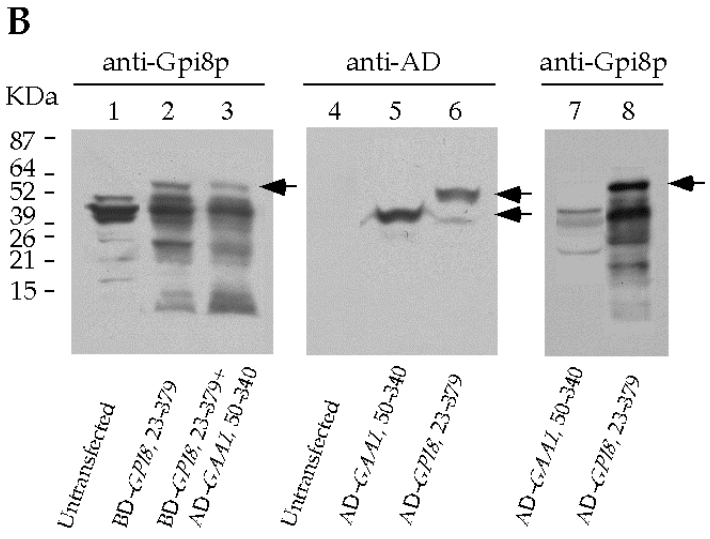

B
anti-Gpi8p
anti-AD
anti-Gpi8p
KDa
1 2 3
4 5 6
7 8
87
64
52
39
26
21
15
Untransfected
BD-GPI8, 23-379
BD-GPI8, 23-379+ AD-GAA1, 50-340
Untransfected
AD-GAA1, 50-340
AD-GPI8, 23-379
AD-GAA1, 50-340
AD-GPI8, 23-379

FIG. 1. **Two hybrid assay showing an interaction between Gpi8p$^{23-379}$ and various fragments of Gaa1p.** A, Constructs specified by the symbol legend were transfected into the yeast strain Y187 and colonies harbouring the desired plasmids were streaked onto agar plates. The picture shows the result of the colony lift filter β-galactosidase assay after 1 hour incubation at 30°C. Recombinant proteins present in the different transfectants are schematically represented near each sector. The lengths of the *GAA1* fragments are indicated. B, Y187 expressing the indicated fusion proteins were cultured overnight at 24°C. Proteins equivalent to 1.5 OD_{600} units of cells were analyzed by 12% SDS-PAGE and probed with rabbit anti-Gpi8p or mouse monoclonal anti-AD. Arrows indicate full length fusion proteins.

II.3.2.2. The hydrophilic parts of Gpi16p and Gpi17p strongly interact directly with each other, and like the hydrophilic part of Gpi8p, show the property of homo-oligomerization.

The putative interactions, with each other or with themselves, of the hydrophilic parts of the four proteins known to take part of the GPI-Transamidase Complex (Gpi8p, Gaa1p, Gpi16p, and Gpi17p), were studied in a two hybrid experiment. This experiment was performed independently to and a few month later than the experiment which show that the hydrophilic, lumenal parts of Gpi8p and Gaa1p interact directly with each other. This can explain the differences observed between the two experiments in the measurement of the blue color apparition in the colony lift filter assay, and of the β-galactosidase levels in the liquid culture assay, for the colonies coexpressing BD-Gpi8p$^{23-379}$ and AD-Gaa1p$^{50-340}$.

In the colony lift filter β-galactosidase assay, colonies coexpressing BD-Gpi8p$^{23-379}$ and AD-Gpi8p$^{23-379}$ became visibly blue within 1h of incubation at RT, colonies coexpressing BD-Gpi17p$^{30-470}$ and AD-Gpi16p$^{30-550}$ within 2h, and colonies

coexpressing BD-Gpi17p$^{30-470}$ and AD-Gpi17p$^{30-470}$ within 3h. Colonies coexpressing BD-Gpi8p$^{23-379}$ and AD-Gaa1p$^{50-340}$, BD-Gpi16p$^{30-550}$ and AD-Gpi16p$^{30-550}$, BD-Gpi16p$^{30-550}$ and AD-Gpi17p$^{30-470}$, became visibly blue between 10 and 20 hours of incubation at RT. We considered that colonies which didn't become blue after 20 hours, show a negative result for the interaction between the corresponding coexpressed proteins. All these results were confirmed by measuring β-galactosidase levels in a liquid culture assay, and for colonies coexpressing all combined proteins (see values in table IV).

GAL4-BD-Fusions \ GAL4-AD-Fusions		GPI8	GAA1	GPI16	GPI17
	- *0*	- *0*	- *0*	- *0*	- *0*
GPI8	- *0*	++ *5.1*	+ *0.24*	- *0.03*	- *0.03*
GAA1	- *0*	- *0*	- *0*	- *0*	- *0*
GPI16	- *0*	- *0*	- *0*	+ *0.12*	+ *0.12*
GPI17	- *0*	- *0*	- *0*	++ *0.25*	++ *0.25*

Table VI. Direct interactions of the hydrophilic parts of the indicated proteins determined by a GAL4-Two-Hybrid system. In the colony lift filter β-galactosidase assay, colonies coexpressing the indicated proteins were streaked onto a selective medium, transferred onto a filter containing X-gal, a substrate for the β-galactosidase. The filter is then incubated at RT and the apparition of the blue colour screened. A strong signal, which appears after 0 to 3h of incubation at RT, is indicated by a double cross (++), a weaker signal (3-20h) by a signle cross (+), and the absence of any signal after 20h by a single bar (-). β-galactosidase levels of corresponding colonies were measured in a liquid culture assay; the measured values, expressed in Miller units, are indicated in italic.

II.3.2.3. A Two-Hybrid-based Model for the Macromolecular organization of the Transamidase Complex

The proposed model is based on the results obtained with the two-hybrid system, which show the direct interaction of Gpi16p with Gpi17p, Gpi8p with Gaa1p, and Gpi8p, Gpi16p, and Gpi17p with themselves. The proposed model also takes into account several other observations which are consistent with the results obtained in the two-hybrid experiment:

* The transamidase complex that we purified in yeast by using a GST-tagged version of Gpi8p, contains Gaa1p and Gpi16p. According the two-hybrid results in which Gpi16p does not interact directly nor with Gpi8p, neither with Gaa1p, the presence in the purified complex of Gpi16p could be promoted by an interaction which needs the presence of both proteins, Gpi8p, and Gaa1p (see Fig.2).

* The purified GPI-transamidase complex migrates in a blue native gel to a corresponding size of 430 to 650 kDa. Since only Gpi8p, Gaa1p, and Gpi16p are found in this purified complex, and taking in account their respective apparent molecular masses of 50, 70, and 79 kDa (taking together, they sum up to about 200kDa), it is conceivable that the 430 to 650 kDa complex contains more then one copy of some of these three proteins.

* The purified hydrophilic part of Gpi8p (II.2.2.2., p.93), loaded onto a native polyacrylamide gel, migrates to apparent masses of 40, 80, and 120 kDa, corresponding respectively to monomers, dimers, and trimers (Fraering, *unpublished*). This observation was confirmed by loading the same sample onto a gel filtration column; the gel-filtration elution profile shows a high narrow peak corresponding to a molecular mass of 40 kDa, preceded at its base by a low large peak, corresponding to molecular masses of dimers and trimers (80 and 120 kDa), thus confirming the homo-oligomerization of Gpi8p.

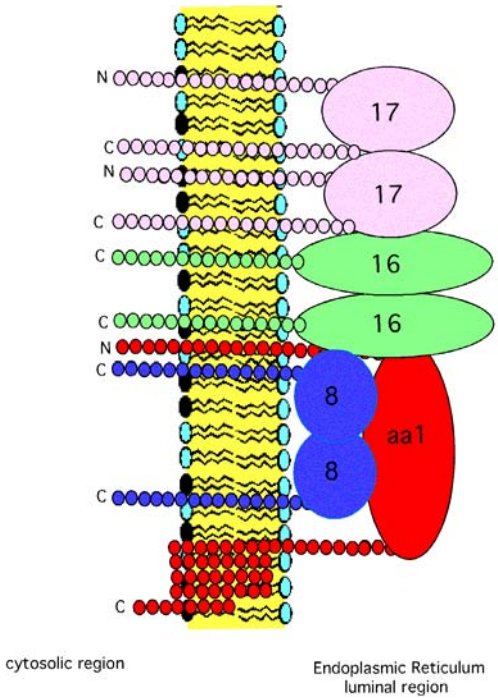


Fig.2. Schematic representation of a Two-Hybrid-based Model for the Macromolecular organization of the Transamidase Complex. The cytosolic C-terminal regions of Gpi8p, Gaa1p, Gpi16p, and Gpi17p, are composed respectively by 14, 14, 38, and 42 amino-acids, whereas the N-terminal cytosolic regions of Gaa1p and Gpi17p contain respectively 19 and 8 amino-acids. Gaa1p, Gpi16p, and Gpi17p show at their C-terminal region a putative ER localization sequence.

II.3.3. Conclusions

Results obtained with the two-hybrid system in the first part of this study point to an interaction between the yeast Gaa1p and Gpi8p. Also with this system, Gaa1p may be expressed above physiological levels, but, fortuitously, Gpi8p was expressed at far lower levels, suggesting that the interaction of these two proteins can occur in a physiological situation. In the two hybrid system the interacting proteins must be sufficiently folded to allow their BD and AD domains to be active, thus making unlikely that they interact merely because of improper folding. In fact, if overexpression is used to demonstrate the

interaction (6), a large proportion of overexpressed proteins may never be engaged in GPI anchoring, and this could lead to the artificial perpetuation of a normally transient interaction. In the same way, Gpi8p and Gaa1p cannot perform their physiological activity when they are expressed in the two hybrid system and thus may perpetuate an otherwise transient interaction. Nevertheless our data seem to favour the view that Gpi8p remains most of the time in close association with other proteins. One argument derives from the fact that Gpi8p forms lacking a C-terminal MSD can functionally replace wt Gpi8p. It has to be taken into consideration that Gpi8p is near to rate limiting for cell growth in wt cells. Since C-terminally truncated Gpi8p versions, expressed at about physiological levels support a normal growth rate, we have to assume that these constructs are efficiently retained in the ER although they are swamped out of the ER when they are overexpressed (II.2.2.1., Fig. 2, p.92). Thus, the ER must contain a limited number of binding sites for Gpi8p, which have sufficient affinity to retain the soluble Gpi8p alleles. These sites may well be provided by Gaa1p.

Results obtained in the second part of this study point to a strong interaction between the yeast Gpi16p and Gpi17p, and a strong interaction of Gpi8p and Gpi17p with themselves, in comparison with the weaker interactions observed between Gpi8p and Gaa1p, and the interaction of Gpi16p with itself. Based on these observations, and in agreement with several arguments mentioned above, a model for the structural and macromolecular organization of the GPI-transamidase complex is proposed. Ohishi *et al*. (2001) also propose a model for the transamidase complex, in which Gpi16p, like in our model, has a central role in the structure of the complex. Their model is based on the analysis of the stability of the four identified components of the GPI transamidase complex, when all 4, or only 3 or only 2 of the differentially tagged components are overexpressed in CHO cells. Our model proposed here has the advantage to show informations about the homo-oligomerization of several components, like Gpi8p, Gpi17p, and Gpi16p.

Both 430 and 650 kDa forms of the purified complex is an argument for the dynamic organization of the complex. Although an interaction of several components of the complex can be demonstrated, it cannot be told at the moment if these proteins remain firmly associated throughout the catalytic cycle or if the normal workings of this complex requires that one, or some, or all of these proteins dissociate at a certain stage.

Finally, the functions of Gaa1p, Gpi16p, and Gpi17p have to be elucidaded. One way consists of understanding how one or more of these proteins are involved in the specific recognition of one or more categories of GPI target proteins.

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II.4. Additional results : *in vitro* reconstitution of the Gpi7p enzymatic activity.

Gpi7 was isolated by screening for mutants defective in the surface expression of GPI proteins (1). *Gpi7* mutants are deficient in YJL062w, named *GPI7*. *GPI7* is not essentiel, but its deletion renders cells hypersensitive to Calcofluor White, indicating cell wall fragility. Several aspects of GPI biosynthesis are disturbed in Δ*gpi7*. An abnormal GPI lipid, called M4, is accumulating in *gpi7*. M4 lacks a phosphodiester-linked side chain, probably an ethanolamine phosphate. 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 ER. 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 (1). The possibility that ethanolaminephosphate corresponds to the added side chain has been considered (1), and to verify this hypothesis, we developed two *in vitro* assays.

II.4.1. Experimental procedures.

Strains

Yeast strains were *Saccharomyces cerevisiae* W303-1B (*MATα ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*), CL3-Abyss-86 (*pra1-1, prb1-1, prc1-1, cps1-3, ura3Δ5, leu2-3,112*), FBY182 (*MATα ade2-1 ura3-1 leu2-3,112 his3-11,15 gpi7 :: KanMX4*).

Purification of His6-tagged Gpi7p.

A GPI7 N-terminal fragment corresponding to nucleotides 1 to 411 of ORF was PCR amplified by using plasmid pBF40 as template and two primers : GPI7-Nt-forwards (5'-TATCGATTAT**GAATTC**ATGAACTTGAAGCAGTTC-3') introducing a EcoRI site, and GPI7-Nt-reverse (5'-TATCGATTAT**CTGCAG**CCAGGAATCGTGC-3') introducing a PstI site. Restriction sites are indicated in bold and nucleotides homologue to GPI7 are underlined. A GPI7 fragment corresponding to nucleotides 412 to 2055 was prepared by digesting the pBF40 plasmid with PstI and BstBI. A GPI7 C-terminal fragment corresponding to nucleotides 2056 to 2493 and containing at its 3' end a HIS6 sequence was PCR-amplified by using plasmid pBF40 as template and the following two primers : GPI7-forwards (5'-TATCGATTAT**TTCGAA**TTTGGTGTCCTT-3') with nucleotides of homology to GPI7 and introducing a BstBI restriction site, and GPI7-reverse (5'-TATCGATTAT**AGATCT**TCAGTGGTGGTGGTGGTGGTGATCAAGAGC GCAAAGGAG-3') with the HIS6 sequence (double underlined) and introducing a BglII restriction site. These PCR DNA fragments were inserted into the multiple cloning site of the plasmid Cup195 digested with EcoRI and BglII, thus yielding the plasmid Cup195-GPI7-HIS6, in which the expression of the tagged protein is placed under the control of the *CUP1* promoter . This plasmid was used to transform the yeast CL3-Abyss strain. Expression of the His6-tagged Gpi7p was induced with 500µM of CUSO4 for 2h at 30°C. The equivalent of 3000 OD600 cells were harvested and resuspended in 40ml of lysis buffer (50mM tris-Hcl, pH8.0, 150mMNaCl, 5mM EDTA, 1mM PMSF, and 1µg/ml of leupeptin, pepstatin, and chymostatin. The cells were lysed by agitation with glass beads at 4°C (MSK homogenizer, 4 runs of 1min/1min in ice), and the crude extract (S6) recovered by centrifugation at 600g/5'/4°C to remove the non lysed cells. The supernatant was centrifuged at 13.000g/15'/4°C and the pellets, corresponding to the microsomes, resuspended in 7 ml of solubilization buffer (SB) (extract S13 in 50mM tris-Hcl, pH 8.0, 150mM NaCl, 1% TX100). After 1h of incubation at 4°C, the solubilized proteins were recovered by centrifugation at 40.000rpm/30'/4°C. The resulting supernatant (S40) was then incubated overnight in batch with 1ml of IMAC Talon resin equilibrated in 15ml of solubilization buffer containing 0.01% of TX100. The resin was then washed first with 10ml of SB+ 0.01% TX100, then with 10ml of SB+ 0.1% deoxy bigCHAP+ 10mM imidazole. The His6-Gpi7p were then eluted with 5ml of SB+ 0.1% deoxy bigCHAP+ 80mM imidazole. The proteins were concentrated by using a centricon 30KD and stored at –80°C after the addition of 15% of glycerol and 1mM of β-mercaptoethanol.

Labeling of cells.

Previously described procedures were used to label cells with (2-^{3}H)Ins (2) and to label microsomes with UDP-(^{3}H) GlcNAc (3). Lipid extracts were analyzed by ascending TLC using 0.2-mm thick silica gel plates with the solvent 10 :10 :3 (chloroform/methanol/water 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.

In vitro reconstitution of the Gpi7p activity.

An aliquot of microsomes (equivalent to 100 μg of proteins) prepared as described from Δ*gpi7* cells (3) were preincubated for 10min at 30°C in presence of the purified Gpi7p, or Gpi8p, and of a mix containing GDP-mannose. After the preincubation, the radioactivity (UDP-(^{3}H) GlcNAc, 6μCi) was added, and the solution incubated at 30°C for 2h. The labeling reaction was stopped by the addition of chloroform:methanol (1:2, v/v), and lipids extracted as described (4). The equivalent of 500.000cpm of each lipid sample were loaded and separated on a TLC.

Reconstitution of proteoliposomes with purified proteins, detergents and lipids.

PC, PE, PS, and PI were purchased from Sigma as chromatographically purified substances. To prepare a lipid stock solution, PC, PE, PS, and PI solutions (each 10mg/ml) were mixed in the ratio 100:25:3:12.5. Half a milliliter of the mixture (5mg of lipid) was added to a 2ml eppendorf tube containing 5mg of deoxyBigCHAP (50μl of a 10% stock solution). DTT(10mM) was added to prevent oxydation. The organic solvent was removed overnight in a speed vac under high vacuum without heating. The residue was dissolved in 250μl of 50mM HEPES-KOH (pH 7.8) and 15% glycerol, with shaking and mild sonication in a watherbath to give a 20mg/ml phospholipid solution.
In pilot experiments, the optimum **lipid to protein ratio** was determined separately for each membrane protein component and for each preparation. The optimum is defined as the lowest amount of phospholipid that results in a quantitative incorporation of the corresponding protein into vesicles (a clear vesicle pellet without aggregated protein).

Approximatively 1μg of phospholipid for 1pmol of each component was needed. In reconstitution assays containing different components, the total amount of phospholipid corresponded to the sum of those needed for each component separately. (5). The molar concentration of detergent (in micellar form) must exceed the molar lipid concentration by at least 10-fold to produce virtually 100% single-walled vesicles, whereas lower **detergent/lipid ratios** were insufficient for complete dispersal of the lipid and led to the appearance of multilamellar liposomes in the final product (6).

Proteoliposomes were formed in a 1ml reconstitution reaction when 60μl phospholipid stock solution (20mg/ml) was mixed with 940μl of purified Gpi7p (10μg/ml) in buffer containing 50mM HEPES-KOH pH7.8, 350mM potassium acetate, 12mM magnesium acetate, 5mM β-mercaptoethanol, 15% glycerol, and 3% w/v deoxyBigCHAP. After the solution became translucent, internal substrate (purified radiolabeled M4) was added and the mix was incubated for 30min on ice. Proteoliposomes were generated by removal of detergent accomplished by overnight incubation in a cold room with 1g of Biobeads SM2 (BioRad) that had been previously equilibrated with 50mM HEPES-KOH pH7.8, 300mM potassium acetate, , 5mM DTT, and 15% glycerol. The fluid phase was separated from the beads, diluted with 5 volumes of ice-cold distilled water, and submitted to centrifugation for 20min at 75.000rpm. The proteoliposomes were finally treated to extract the lipids.

Reconstitution of proteoliposomes with labeled PE

6μCi of labeled PE were evaporated in the speed vac and resuspended in 100 μl of buffer containing 50mM HEPES-KOH pH7.8, 1mM DTT, 15% glycerol, and 3% deoxyBigCHAP. 100μg of purified Gpi7p and deoxyBigCHAP (final concentration=3%) were added to this mix, and incubated for 30min at 4°C. The cold lipid preparation, extract from Δ*gpi7* cells accumulating M4, was resuspended in the PE-Gpi7p mixture ; deoxyBigCHAP was added to a final concentration of 4%. The final mixture was incubated at 4°C for 30min, centrifuged at 40.000rpm/1h/4°C. The detergent phase was recovered, the radioactivity in this phase measured, and proteoliposomes produced by incubating this detergent phase overnight with SM2 Biobeads. The proteoliposomes were incubated for 2h at 30°C and lipid extracted for analysis.

II.4.2. Results

II.4.2.1. The His6-tagged form of Gpi7p conserves its enzymatic activity.

As can be see in Fig.1, lanes 3 and 4, transfection of Δ*gpi7* cells with a plasmid harbouring a HIS6-tagged form of GPI7,almost completely cured the accumulation of M4 observed in the Δ*gpi7* cells (Fig1, lane 2). This demonstrates that the His6-tagged form of Gpi7p is fully functional and conserves the same enzymatic activity observed in Δ*gpi7* cells transfected with a plasmid harbouring the wt GPI7 (1).

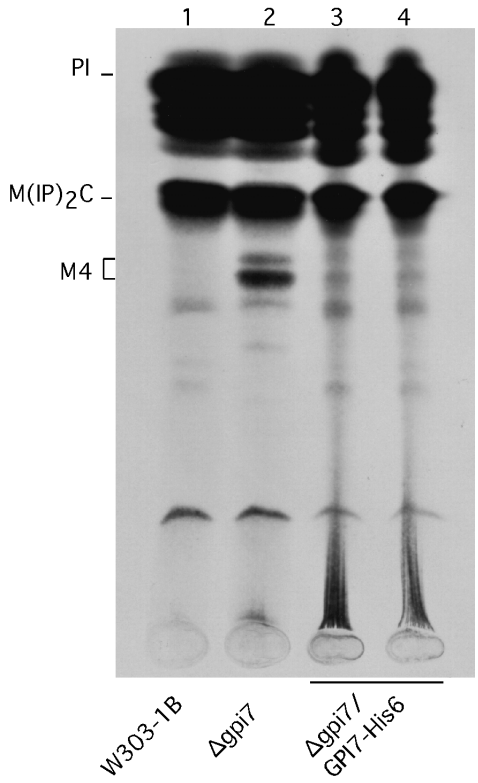


Fig.1. Complementation of Δ*gpi7* cells by His6-tagged Gpi7p. Exponentially growing WT (lane1), Δ*gpi7* (lane 2), and Δ*gpi7* cells transfected with two different clones of plasmid Cup195-GPI7-HIS6 (lanes 3-4) were radiolabeled at 37°C with (^{3}H)Ins (2μCi/A600), and desalted lipid extracts were analyzed by TLC and fluorography. The same amount of radioactivity was spotted in each lane.

II.4.2.2. Purification of the His6-tagged form of Gpi7p

The His6-tagged-form of the protein was overexpressed in the CL3-Abyss strain, a selected strain for the overexpression of recombinant proteins in yeast, mutated in several enzymes of the proteasome. The CL3-Abyss strain was transfected with a plasmid harbouring a *GPI7* fused at its 3' end with a HIS6 tag, and placed under the control of the copper inducible CUP1 promoter (7). Gpi7p is a 830-amino acid long membrane protein with about 9-11 putative transmembrane domains. Gpi7p was shown to be associated with membranes since it could be sedimented by ultracentrifugation (1). The extraction conditions of the overexpressed His6-tagged Gpi7p were analyzed by treating the membranes with several detergents. As can be see in Fig.2, TritonX100 and Digitonin are both able to solubilize with the same effectiveness, three forms of Gpi7p with apparent molecular masses of 182, 120, and 84 kDa. Chaps and Octyl-glycopyranoside are much less effective in the solubilization of the His6-tagged Gpi7p (Fig.2, panel B).

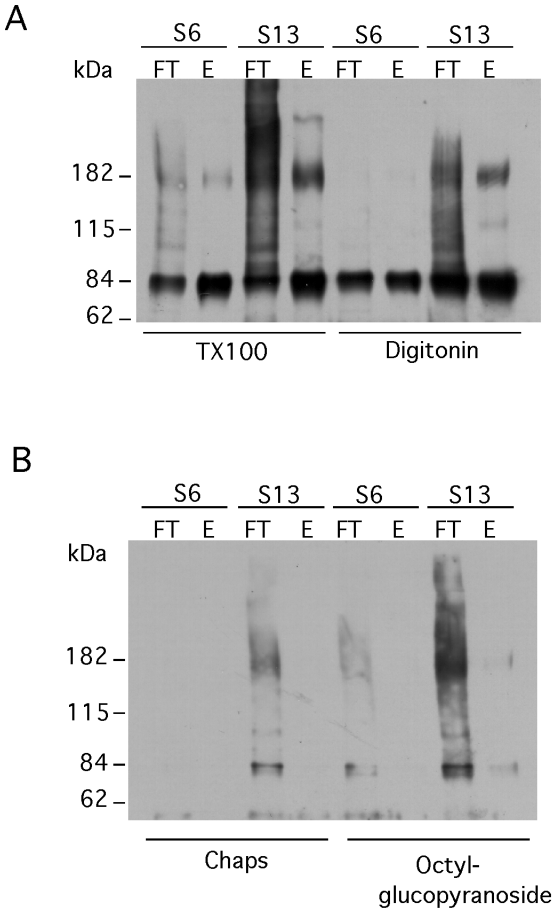


Fig.2. Purification of His6-tagged Gpi7p : solubilization assays. Microsomes were prepared by breaking CL3-Abyss-86 cells transfected with the Cup195-GPI7-HIS6 vector, in which the expression of the His6-tagged Gpi7p was induced with 500µM of CUSO4 for 2h at 30°C. After centrifugation at 600g/5'/4°C, supernatant and pellet (P6) were recovered ; the supernatant was centrifuged at 13.000g/15'/4°C, and the pellet recovered (P13). Pellets (P6 and P13) were then resuspended in different solubilization buffers containg 1% of different detergents : TX100, Digitonin, Chaps, or octyl-glycopyranoside. After 1h of solubilization at 4°C, samples were centrifuged at 45.000g/45'/4°C, and supernatants loaded onto a Talon affinity column. After 1h of binding at 4°C, the resin was centrifuged at 700g/4'/4°C and supernatants S6 and S13 recovered (FT=Flowthrough). The resin was washed and the proteins eluted with 160mM of Imidazole+0.2% of the corresponding detergent. The eluted proteins (E=elution) were separated by SDS-PAGE, and probed with affinity purified anti-Gpi7p antibodies.

The 84 kDa form of Gpi7p (Fig.2) may be an ER form or a cytosolic form caused by the overexpression of the His6-tagged Gpi7p. As also can be see in Fig.2, the most material that reacts with affinity purified anti-Gpi7p antibodies is in the unbound fraction ; this material could correspond to Gpi7p degradation products which lost the Hi6-tag, or to His6-tagged Gpi7p in which the Hi6-tag is masked by the conformation of the protein, after or before its glycosylation.

A large scale purification protocole detailed under Experimental and Procedures was used to purify the His6-tagged Gpi7p . This protocole is based on a Triton X100 extraction from membranes of the overexpressed proteins, and on the His6 tag affinity purification. As can be see on Fig.3, lane 7, five major bands appear after separation and staining of the purified proteins (Fig.3, arrows). These bands correspond to proteins with apparent molecular masses ranging from about 220 to 84 kDa. The characterization of the native Gpi7p showed that affinity purified antibodies against the N-terminal, hydrophilic part of the protein, recognize various glycoforms of Gpi7p, with estimated molecular masses ranging from 83 to 230 kDa , and that the proteins can be deglycosylated to an apparent molecular mass of 86kDa by treatment with Endoglycosidase H (1). These results are more or less in agreement with the purified His6-tagged Gpi7p, indicating that these His6-tagged proteins are also able to be glycosylated in the secretory pathway.

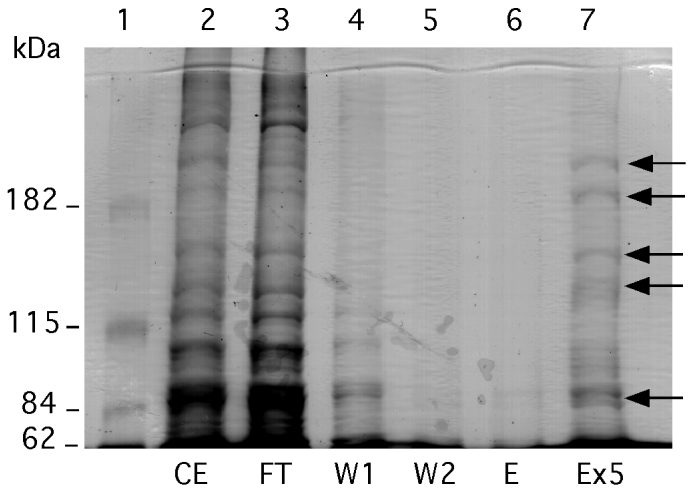
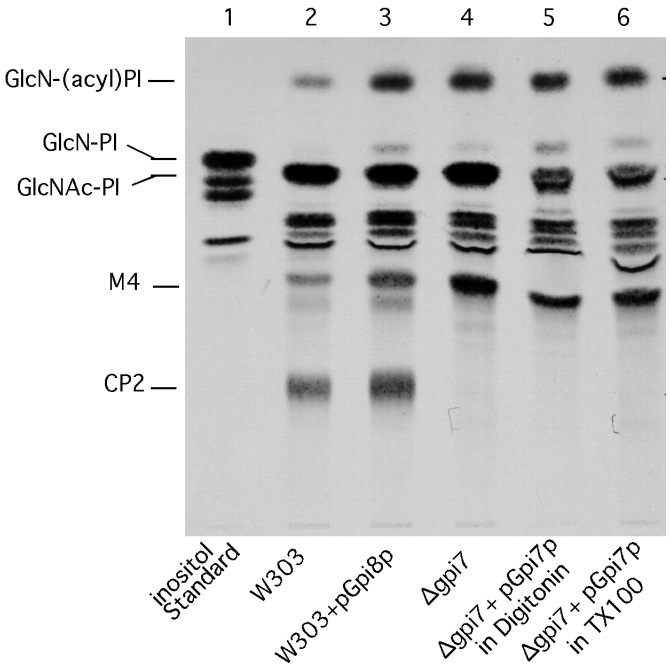


Fig.3. Affinity purification of His6-tagged Gpi7p. His6-Gpi7p were prepared as described in the Experimental Procedures. Briefly, the solubilized proteins (CE=crude Extract) were loaded onto the affinity column and the flowthrough discarded (FT). The column was washed twice with two different buffers (W1 and W2), the bound proteins eluted with an imidazole-containing buffer (E) and concentrated five times with a centricon device (Ex5). Proteins were separated by SDS-PAGE, and revealed by Coomassie-blue staining.

II.4.2.3. In vitro reconstitution of the Gpi7p activity

Based on the analysis of M4, an abnormal GPI lipid accumulating in *gpi7*, it was proposed that Gpi7p adds a side chain onto the GPI core structure (1). After the discovery of an additional EtN-P on Man1 of CP2 (8), the possibility that Gpi7p adds an EtN-P onto M4 was considered. To verify this hypothesis, two *in vitro* assays were developped. As see on Fig.4A, lane 4, microsomes of Δ*gpi7* labeled with ^{3}H UDP-GlcNac synthesize M4, and not CP2, whereas microsomes of WT synthesize CP2 (Fig4A, lane 2). The first *in vitro* assay is based on the hypothesis that the addition of purified recombinant Gpi7p to microsomes of Δ*gpi7* , before the labeling with ^{3}H UDP-GlcNac, could maybe restore the synthesis of CP2. The experiment was performed, and as it can be see on Fig4A, lanes 5 and 6, or on Fig4B, lane 3, no significant increase in the CP2 synthesis can be observed, after the addition of purified Gpi7p to Δ*gpi7* microsomes.

A


1
2
3
4
5
6
GlcN-(acyl)PI
GlcN-PI
GlcNAc-PI
M4
CP2
inositol Standard
W303
W303+pGpi8p
Δgpi7
Δgpi7 + pGpi7p in Digitonin
Δgpi7 + pGpi7p in TX100

B

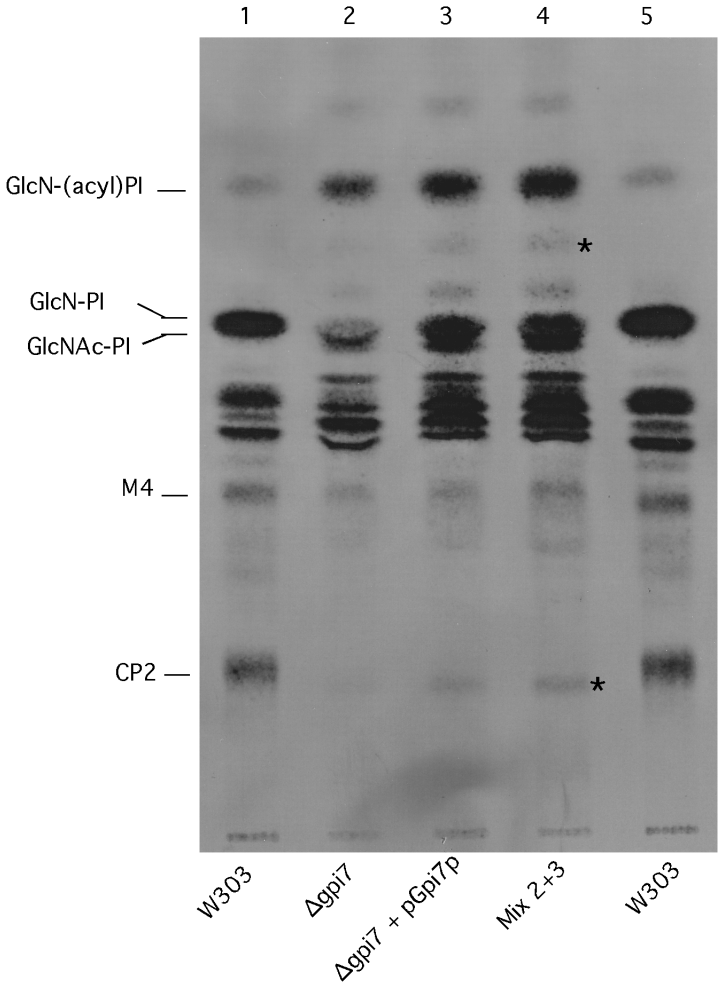

1
2
3
4
5
GlcN-(acyl)PI
GlcN-PI
GlcNAc-PI
M4
CP2
*
*
W303
Δgpi7
Δgpi7 + pGpi7p
Mix 2+3
W303

Fig.4. *In vitro* reconstitution of the Gpi7p activity. A, B, Microsomes were prepared as described from the indicated strains and preincubated for 10' at 30°C in presence of GDP-mannose and the indicated purified proteins. 6 μCi of ^{3}H UDP-GlcNac was then added and the tubes were incubated for an additional 2h at 30°C. The reaction was stopped in chloroform/methanol 1 : 2 (v/v) and the lipids were extracted as described in experimental procedures. The equivalent of 500.000cpm of each assay was loaded onto a TLC. Lipids which appear after the addition of purified Gpi7p are marked with an asterisk (B, *).

The second *in vitro* assay involved the preparation of proteoliposomes containing lipids extracted from Δ*gpi7* cells (accumulating M4), radiolabeled PE, and purified Gpi7p. The working hypothesis was that in these proteoliposomes, purified Gpi7p recognize PE as a donor of EtN-P, and promote the transfert of the labeled EtN-P onto cold M4 lipids, thus yielding in the formation of radiolabeled CP2 lipids. The experiment was performed, but no apparition of significant labeled CP2 could be observed.

II.4.3. Conclusions

The hypothesis that Gpi7p adds an EtN-P onto M4 was not confirmed with our *in vitro* microsomal reconstitution assay. An important point in such an assay is the proper folding of the added, purified protein. It has been shown that in a cell lysate, Gpi7p is rapidly degraded by an endogenous protease (1) ; in the *in vitro* assay, the purified protein becomes possibly degraded, or becomes unable to be actively folded. In the cell, Gpi7p is localized in the plasma membrane and has 9-11 transmembrane domains; force the purified protein to enter into a membrane in a properly folded structure could be a limiting factor in our in vitro assay. We then started the development of another in vitro assay, based on the reconstitution of proteoliposomes with purified protein components, lipids and detergents. The formation of proteoliposomes should favour the integration in a properly folded structure of the purified Gpi7p. Proteoliposomes were formed when cold lipid extracts from Δ*gpi7* cells (containing M4) were mixed, in a buffer containing 3% deoxyBigCHAP, with purified Gpi7p and with labeled PE, the ethanolamine-phosphate donor in the cell (8). Proteoliposomes were generated by removal of detergent. These were incubated at 30°C, and lipids extracted and analyzed. The synthesis of labeled CP2 lipids, formed by the addition of a labeled ethanolamine-phosphate to cold M4, was never

observed in the proteoliposomes *in vitro* assay. Our results suggest that Gpi7p is not able to add a side chain onto the M4 lipid, or that it is not the only component required for this process. We can easily imagine a sequential, well ordered, reaction in the biosynthesis of the complete GPI precursor in which Gpi7p acts before the formation of M4. If this is the case, the purified Gpi7p can not recognize M4 as a substrate, and can not transfer any side chain onto this lipid. The specificity of the substrate of Gpi7p is not known and should be an important point in the reconstitution of its enzymatic activity. The question of the function of Gpi7p still be open and is an interesting field in research.

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III. FINAL CONCLUSION

Che *et al*. (1998) found a motif **His**-Gly-spacer-Ala-**Cys** which can be recognized in a clan called CD comprising the families of Gpi8 (C13), Caspase-1 (C14), Clostripain (C11), and GingipainR (C25). Alignements of the catalytic sites of each family revealed in all four families a block of four hydrophobic amino-acids closely preceding each of the catalytic residues. We performed a topological search of the yeast and Human Gpi8p structure with GORIV and PREDATOR which revealed topological similarities between these proteins and gingipainR and Caspase-1. A closer look showed that the two blocks of hydrophobic amino-acids immediately preceding each of the catalytic residues are included in β-sheet structures conserved in yGpi8p (a.a. 150-155, a.a. 190-194), hGpi8p (a.a. 156-162, a.a. 198-205), and RgpB (a.a. 204-210, a.a. 236-243). According to these observations, Gpi8ps can be associated, like RgpB, with an open β-sheet enzyme family having a characteristic active center localized in a crevice, just outside the carboxyl end of two conserved β-sheets. In each family, an α-helix is shown to be localized between the conserved β-sheets ; in RgpB, this helix (h7) is smaller (7a.a.) in comparison with the same helices in yGpi8p (h5, 25a.a.), and in hGpi8p (h6, 22a.a.). Another significant structural similarity between Gpi8ps, RgpB, and Caspase-1 is the presence of a conserved α-helix, helix h5 in RgpB (a.a. 162-179), helix h5 in hGpi8p (a.a. 126-137), and helix h4 in yGpi8p (a.a. 122-130). The analysis of these conserved helices shows the existence of a consensus motif Glu-Asn-$(X)_{4-5}$ Lys-Thr. The high resolution structure of RgpB revealed a catalytic domain formed by the N-terminal 351 residues, and an IgSF-like domain made by the last 84 C-terminal residues. Gpi8ps can also be characterized by two separated domains : a N-terminal catalytic domain, and a C-terminal domain with an α/β structure, which differs with the all β-structure of the IgSF-like domain of RgpB. The C-terminal domain of Gpi8ps, like RgpB, is dispensable for the catalytic activity of these proteins, as demonstrated by the use of mutated alleles of Gpi8ps lacking the C-terminal region (Fraering, *in preparation* ; Kinoshita *et al*., 2000). Meyer *et al*. (2000) has shown that the region including Ser60 and the five preceding amino-acids is important for the activity of the yGpi8p. The topological search performed here show that this Ser60 region is included in the h3 α-helix, but we can not propose any explanation about how this region is involved in the catalytic organization of the protein. Eichinger *et al*. (1999) proposed that

the Glu152 residue, placed immediately down stream of the S5 β-sheet of RgpB, stabilizes the His211 imidazole in its protonated form, thus promoting the C-N break in the bound substrate (I.7.1.4.3., p.50). No evident equivalent amino-acid residue has been found in Gpi8ps from our topological similarity search. All these structural similarities observed between Gpi8ps, RgpB, and Caspase-1, promote the classification of Gpi8p as a classical α/β structural protein formed by two distinct domains : a N-terminal catalytic domain with a characteristic active diad His-Cys, two residues preceded by conserved β-sheets and surrouding a large α-helix, and a C-terminal non-catalytic domain.

Up to now, no proteolytic activity could be found with purified Gpi8p in an in vitro system. After solubilization in 1.5 % digitonin and separation by blue native polyacrylamide gel electrophoresis, a system which allows a rapid assessment of the state of the GPI transamidase complex under various conditions, Gpi8p is found in 430 to 650 kDa protein complexes. These complexes can be affinity purified and the subunits we identify are Gpi8p (50kDa), Gaa1p (70kDa) and a further component, Gpi16p (YHR188c, 79kDa). Together the subunits sum up to about 200kDa. It is possible that the 430 to 650 kDa complex contains more then one copy of some of these three proteins and/or contains yet other non-identified subunits which were not stained by silver nitrate. Gpi16p is an essential N-glycosylated transmembrane glycoprotein. Its bulk resides on the lumenal side of the ER, it has a single C-terminal transmembrane domain and a small C-terminal, cytosolic extension with an ER retrieval motif. Depletion of Gpi16p results in the accumulation of the complete GPI lipid CP2 and of unprocessed GPI precursor proteins. This demonstrates unequivocally that Gpi16p is not required for the biosynthesis of GPI lipids but rather for their attachment to GPI proteins. Although the functional importance of Gpi16p, as well as Gaa1p and Gpi8p for the attachment of GPI lipids to newly made GPI proteins in the ER is beyond doubt, much remains to be learned about the exact function of these proteins. Recently, Ohishi *et al.*, (2001) purified the transamidase complex in human cells and isolated an additional subunit, PIG-S , which has a homolog in yeast, called GPI17 (YDR434W). Gpi17p is predicted to be an essential N-glycosylated transmembrane glycoprotein. Its bulk resides on the lumenal side of the ER, and two transmembrane domains are predicted near the N- and C-termini. Depletion of Gpi17p results in the accumulation of the complete GPI lipid CP2.

A *GAL4*-based two hybrid system was used to verify the binding of Gpi8p to Gaa1p, and to study the macromolecular organization of the GPI-transamidase Complex. Results

obtained in this study point to a strong interaction between the yeast Gpi16p and Gpi17p, and a strong interaction of Gpi8p and Gpi17p with themselves, in comparison with the weaker interaction observed between Gpi8p and Gaa1p, and the weaker interaction of Gpi16p with itself. The hypothesis which put forward that the 430 to 650 kDa complex contains more then one copy of some of the three proteins is here confirmed. We propose also a model for the macromolecular organization of the transamidase complex, which can be compared with the model proposed by Kinoshita *et al*. (2001). Both models predict a central role for the Gpi16p, with additional informations about the homo-oligomerization of several components proposed by the two-hybrid based model. Finally, Gpi16p was found in the yeast protein database (YPD) to interact in a two-hybrid system with Yjr015p, Pho89p, and Ynl092p. Pho89p, predicted as a membrane protein with 7 transmembrane domains, shows similarities with phosphate transporters, whereas Ynl092p contains a motif conserved in S-adenosylmethionine-dependent methyltransferase. As shown in Results I, Fig.5B, Δ*yjr015* cells did not accumulate GPI precursor lipids. Further studies are required to definitely rule out a role of this protein in GPI anchor addition, and to verify if Pho89p, and/or Ynl092p are involved in the same processus.

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INTERNET SITES

Yeast genome and proteome

MIPS:	http://www.mips.biochem.mpg.de/proj/yeast
MIPS Eurofan	http://websvr.mips.biochem.mpg.de/proj/eurofan/index.html
SGD	http://genome-www.stanford.edu/Saccharomyces
YPD	http://www.proteome.com/databases/index.html

Yeast deletion strains

Euroscarf	http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/col_index.html
Research genetics	http://www.resgen.com/
Stanford yeast deletion database	http://www.stanford.edu/group/yeast_deletion_project/Enter_DB.html
Yeast genetic stock center at ATCC	http://www.atcc.org/L2.cfm?Lexp2=2&Lexp3=2&B=3&Lexp4=14&Lexp5=1

Vectors & primers

Plasmid databank	http://www.geocities.com/HotSprings/Spa/3013/plasmid.html
Vector database	http://vectordb.atcg.com/
Research genetics	http://www.resgen.com/

Gene and protein sequences

SRS	http://www.expasy.ch/srs
SwissProt	http://www.expasy.ch/sprot
PIR (Protein Information Center for protein sequences)	http://www.bis.med.jhmi.edu/Dan/fields/pir.form.html
MIPS	http://www.mips.biochem.mpg.de/
NCBI	http://www.ncbi.nlm.nih.gov/genbank/query_form.html
EMBL	http://www.ebi.ac.uk/embl/
DNA Data Bank of Japan	http://www.ddbj.nig.ac.jp/
Flybase	http://flybase.bio.indiana.edu/
AceDB	http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml

Protein sequence analysis

Expasy	http://www.expasy.ch/
PROPSEARCH	http://www.infobiosud.univ-montp1.fr/SERVEUR/PROPSEARCH/propsearch.html
PSORT	http://psort.nibb.ac.jp/form.html
Expasy proteomic tools	http://www.expasy.ch/tools/

Protein interactions

Two hybrid Interactions	http://www.fccc.edu:80research/labs/golemis/InteractionTrapInWork.html)

Sequence comparison

BLAST2.0 at SGD	http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd
BLAST2.0 at SIB	http://www.ch.embnet.org/software/frameBLAST.html?
NCBI BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
BLAST at ProDom	http://protein.toulouse.inra.fr/prodom/doc/blast_form.html

Multiple sequence alignements

Clustalw	http://www2.ebi.ac.uk/clustalw/
Multiple sequence Alignement	http://www.bork.embl-heidelberg.de/Alignement/alignement.html
Blocks Multiple Alignement Processor	http://www.blocks.fhrcrc.org/process_blocks.html
BOXSHADE	http://www.ch.embnet.org/software/BOX_form.html

Pattern, motifs, domains and profiles

Expasy proteomic tools	http://www.expasy.ch/tools/
Pratt at EBI	http://www2.ebi.ac.uk/pratt/
Emotif	http://motif.stanford.edu/projects.html
ISREC Profile homepage	http://www.isrec.isb-sib.ch/profile.html
ISREC ProfileScan server	http://www.ch.embnet.org/software/PFSCAN_form.html
Pfam	http://pfam.wustl.edu/

2D- and 3D structure

Expasy tools	http://www.expasy.ch/tools/
S3D	http://genome-www.stanford.edu/Sacch3D/domains/
Protein Data Base (PDB)	http://www.rcsb.org/pdb/
SCOP (Structural Classification of Proteins	http://scop.protres.ru/

GPI proteins

Sequence analysis of GPI-Anchored proteins on the Proprotein level	http://www.embl-heidelberg.de/~beisenha/GPI/gpi.html
Big-PI Predictor	http://mendel.imp.univie.ac.at/gpi/gpi_server.html

Molecular biology servers

Amos Bairoch's links for Life scientists	http://www.expasy.ch/alinks.html
Pedro's Molecular Biology Research tools	http://www.public.iastate.edu/~pedro/research_tools.html
PubMed	http://www.ncbi.nlm.nih.gov/PubMed/

DECLARATION FOR THE FACULTY

Patrick Fraering
Rue de Kaisa 6
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SUISSE

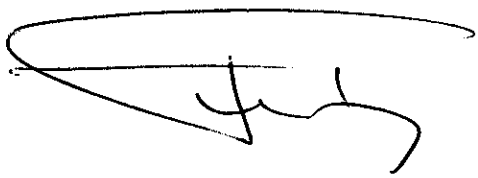
Fribourg, le 09 Octobre 2001

Aux personnes concernées,

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 avoir rédigé ma thèse intitulée " Functional analysis of the yeast *Saccharomyces cerevisiae* Gpi8 protein and characterization of the purified GPI-transamidase complex" sur la base d'un travail personnel et sans aucune aide illicite.



Patrick Fraering

CURRICULUM VITAE

Patrick Fraering

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Birthday and place:	15 june 1970, Selestat (Bas-rhin, France)
Nationality:	French
Family status:	Single
Home address:	rue de Kaisa 6, 1726 Farvagny-le-Grand
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Professional Address:	Institute of Biochemistry
	University of Fribourg
	Rue du musee 5
	1700 Fribourg-Switzerland
E-mail Address:	patrick.fraering@unifr.ch
Languages:	French, English and German.

School and academic degrees:

1989 : General Certificate of Education (Baccalaureat) in the Dr Koeberle High School of Selestat-France.

1989-1991: University Diploma of Technology (DUT) in Applied Biology (biological and food Industries), Institute of Technology , University Louis Pasteur, Strasbourg-France.

1992: National service.

1993: License of Biochemistry, option Genetics and General Microbiology, University Louis Pasteur of Strasbourg.

1994: Master's degree of Biochemistry, option Molecular Virology and Applied Microbiology, University Louis Pasteur of Strasbourg

1995: Diploma of thorough studies (DEA) in Molecular and Cellular Biology, Institute of Molecular and Cellular Biology (IBMC, director Prof.B. Ehresmann), National Scientific Research Center (CNRS-UPR9002-France).

Works: Yeast mitochondrial phenyl-alanyl tRNA synthetase and its interaction with the corresponding tARN : overexpression, purification and cristallisation studies.

1996: Preparative year to the competition examination to become a Higher education Master in Biochemistry (Aggregation), Institute of Master Education, University of Strasbourg.

1997-2001 : Ph.D. student in the lab of Prof. Conzelmann, Institute of Biochemistry, University of Fribourg (Switzerland).
Works: Gpi anchoring in *Saccharomyces cerevisiae* : Mecanisms of Gpi anchors attachment on target proteins.

Seminars and conferences:

1995: Procop workshop "tRNA-tRNA synthetases", Mont Saint-Odile, France.
1998: 2nd Cell Biology Symposium of the MDC on Protein Transport and Stability, Max Delbruck Center for Molecular Medicin, Berlin. Abstract and Poster presentation.
1998: Swiss Yeast Meeting, Fribourg. Abstract and Poster presentation
1998: 30st Annual Meeting of the USGEB/USSBE.
1999: FEB'S 99. 26th meeting of the Federation of European Biochemical Societies, Nice.
1999: 31st Annual Meeting of the USGEB/USSBE.
2000: Swiss Yeast Meeting, Basel.
2001: ASBMB Annual Meeting, Orlando Florida. Abstract and Poster presentation

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Teaching Experience:

1997-2001: University of Fribourg, Institute of Biochemistry, Practical Courses for 3rd year students in Biochemistry (License), students in Biology, Pharmacy and Medicine.
1999-2000: Director of Diploma thesis of Andre Haenni
2000-2001: Director of Diploma thesis of Yannick Frische.

Additional training:

1999: Strategies for enhanced solubility an yield of proteins expressed in E.coli.Institute of pharmacology and toxicology. Lausanne.
2000: European Electophoresis Tour 2000. FMI.Basel.

Membership in Scientific and Professional Associations:

Since 1997	Swiss Society of Biochemistry (SGB)
Since 1997	Swiss society of Experimental Biology (USGEB)
Since 1999	Member of the University council, University of Fribourg
Since 2000	Swiss Society of Cell Biology, Molecular Biology and Genetics (ZMG)

Scientific Publications:

Patrick Fraering, Isabella Imhof, Urs Meyer, Jean-Marc Strub, Alain van Dorsselaer and Andreas Conzelmann.(2001).The GPI transamidase complex contains Gpi8p, Gaa1p and Gpi16p. Molecular Biology of the Cell, Vol.12, 3295-3306, October 2001.

Patrick Fraering and Andreas Conzelmann.(2001) Functional and Structural Analysis of Yeast Gpi8p, an Essential Component of the Yeast GPI Transamidase Complex. In preparation.

Urs Meyer, Isabella Imhof, **Patrick Fraering**, and Andreas Conzelmann.(2001).
The GPI signal sequence of human placental alkaline phosphatase is not recognized by the yeast GPI anchoring machinery. In preparation.