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***GPI anchor attachment in yeast:  
analysis of Gpi8p, the putative catalytic  
subunit of the GPI:protein transamidase***

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***Für Olivia,  
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in lieber Erinnerung an Anita***

## STRUCTURE OF THE MANUSCRIPT

In eucaryotic cells, many glycoproteins are attached to the external surface of the plasma membrane by glycosylphosphatidylinositol (GPI) anchors covalently linked to their carboxy terminus. GPI-anchored proteins have been discovered to play a crucial role in development, in the proper functioning of the immune system and in parasite pathogenesis.

Five years ago, two yeast proteins, namely Gaa1p and Gpi8p, had been discovered and were - because of the deficiency in GPI anchor attachment of the corresponding mutants - advocated to be subunits of the putative GPI:protein transamidase, the yet unidentified enzyme complex responsible for the transfer of a newly made complete GPI precursor glycolipid onto the immature GPI protein precursor.

The document at hand tries to present recent data on the *Saccharomyces cerevisiae* protein Gpi8p and represents the summary of results I have obtained as PhD student in the lab of Prof. Dr. Andreas Conzelmann.

This thesis manuscript starts with an introduction designed as a small journey through the biochemical landscape of GPI anchoring. During this journey, general biochemical concepts of a living cell and biochemical pathways which influence GPI anchoring are described side-by-side and should help to understand that GPI anchor addition is narrowly woven into the immense wealth of different biochemical reactions occurring simultaneously with an incredibly high accuracy within a cell. GPI anchoring of several eucaryotic organisms - although focussing preferentially on the yeast *Saccharomyces cerevisiae* system - is explained by providing information on GPI anchor biosynthesis, GPI anchor attachment, transport of GPI-anchored proteins, functions of GPI glycolipids and GPI-anchored proteins as well as medical aspects. The process of GPI anchor attachment is described in detail, whereas all other aspects of GPI anchoring are expressed in a short reviewing manner.

The Results part describes published data, results which still have to be substantially completed before publication and additional experimental data which help to define working concepts. Every separate result chapter is divided into an Introduction, a Result, a Discussion and an Experimental Procedures or Methods paragraph. In this part, data mainly on Gpi8p, the putative catalytic subunit of the GPI:protein transamidase are presented and are supplemented with data on other proteins predicted or shown to be involved in the yeast GPI metabolism such as putative remodelases and a database for yeast GPI proteins.

In the Final Discussion part, results are discussed and propositions for future experiments, made in the discussion part of the various Result chapters, are shortly summarized in the Outlook chapter.

Part 5 describes the general Material and Methods which were used to perform all experiments unless given already in the Experimental Procedures part of the different chapters. The Experimental Procedures paragraphs of separate Result chapters describe special techniques used only for the corresponding Result chapter or contains supplements or changes compared to the general protocols listed in Materials and Methods.

Part 6 and 7 include all cited references or Web pages, respectively.

The Annex part contains lists of data too extensive to be put into the main text. Here included is a list of all known and predicted GPI proteins in *S. cerevisiae*.

The document closes with a list of all Tables and Figures shown, an overall index which references the whole document including papers, and my curriculum vitae.



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As an unexperienced student I started my diploma thesis in the laboratory of Prof. Dr. Andreas Conzelmann. I was rapidly fascinated about his scientific excellence, his very supportive criticism and his enormous kindness. He let me learn a lot during frequent discussions, which were most often very long, sometimes short, but always hold in a way to come up with new, creative ideas or to think up new approaches to enable and ease the exploration of our basic scientific questions. He joined his concise and precise criticism with a remarkable, gentle touch of positivity and humor. All this let me skip my previous plans to perform my PhD studies at another university. And I did not wrong, he did not loose all these excellent qualities and capacities over the years of my PhD studies. Beyond his permanent support for my work in the lab, he allowed and even motivated me to perform several courses outside of our institute and our university. I would like to thank Prof. Dr. A. Conzelmann for giving me the possibility to perform my PhD thesis on the very attractive field of secretion, for his permanent support, his excellent expertise and guidance, for his enthusiasm and for his time he attended to me. Thank you.

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## ABBREVIATIONS

AP	alkaline phosphatase
BSA	bovine serum albumine
CDP-EtN	cytidine diphosphate ethanolamine
Cer	ceramide
CFW	calcofluor white
Chx	cycloheximide
CoA	coenzyme A
COP	coatamer protein
CP	complete precursor
cpm	counts per minute
CRD	cross reacting determinant
DAF	decay accelerating factor
DAG	diacyl glycerol
DFP	diisopropylfluorophosphate
Dol	dolichol
DPM	dolichol phosphomannose
DPAP A	dipeptidyl aminopeptidase A
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetate
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
EtN	ethanolamine
FCS	fetal calf serum
Gal	galactose
GIPL	glycophosphoinositol lipids
Glc	glucose
GlcN	glucosamine
GlcNAc	N-acetyl glucosamine
GPI	glycosyl phosphatidylinositol
GPI-PLC	GPI-specific phospholipase C
GPI-PLD	GPI-specific phospholipase D
hGH	human growth hormone
HPLC	high performance liquid chromatography
Ins	<i>myo</i> -inositol
IPC	inositolphosphoceramide
JBAM	Jack bean $\alpha$ -mannosidase
LCB	long-chain base
LPG	lipophosphoglycan
LPPG	lipopeptidophosphoglycan
M(IP) <sub>2</sub> C	mannosyl diinositolphosphoceramide
Man	mannose
min	minute(s)
MIPC	mannosyl inositolphosphoceramide
MIPS	Munich Information Center on Protein Sequences
myr	myriocin
NCAM-120	neural cell adhesion molecule of 120 kDa

NEM	N-ethyl maleimide
NGF	nerve growth factor
NSF	N-ethylmaleimide-sensitive fusion factor
OD	optical density
P	phosphate
Pal	palmitate
PARP	<i>Trypanosoma brucei</i> procyclic acid repeat protein (= procyclin)
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI-PLC	PI-specific phospholipase C
PLAP	placental alkaline phosphatase
PMSF	phenyl methanesulfonyl fluoride
PNH	paroxysmal nocturnal hemoglobinuria
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
rpm	rotations per minute
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i> (baker's yeast)
SDS	sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SGD	<i>Saccharomyces cerevisiae</i> genome database
SNAP	soluble NSF attachment membrane protein
SQL	structured query language
SRP	signal recognition particle
TAP	T cell activating protein
TGN	<i>trans</i> -Golgi network
TLC	thin layer chromatography
TRIS	tris-(hydroxymethyl)-aminomethane
ts	temperature-sensitive
t-SNARE	SNAP receptor on the target membrane
Ub	ubiquitin
UDP-GlcNAc	uridine diphosphate <i>N</i> -acetyl glucosamine
URL	uniform resource locator
VSG	variant surface glycoprotein
v-SNARE	SNAP receptor on the vesicle membrane
VTC	vesicular tubular cluster
wt	wild type
www	world wide web
YPD	Yeast Protein Database



**Genes**

<i>ALG</i>	<u>a</u> sparagine <u>l</u> inked <u>g</u> lycosylation
<i>CPY</i>	carboxypeptidase <u>Y</u>
<i>DPM</i>	<u>d</u> olichol <u>p</u> hoshate <u>m</u> annose synthase
<i>DER</i>	<u>d</u> egradation in the <u>ER</u>
<i>END</i>	<u>e</u> ndocytosis
<i>ERD</i>	<u>ER</u> <u>r</u> etention defective
<i>ERG</i>	<u>e</u> rgosterol biosynthesis
<i>GAA1</i>	<u>G</u> PI <u>a</u> ncor <u>a</u> ttachment gene 1
<i>GAS1</i>	<u>g</u> lycophospholipid- <u>a</u> nchor <u>e</u> d <u>s</u> urface protein 1 (named before <i>GGP125</i> ( <u>G</u> PI-anchored <u>g</u> lycop <u>p</u> rotein of 125-kDa))
<i>GPI8</i>	<u>G</u> PI biosynthesis gene 8 encoding the putative GPI:protein transamidase
<i>KAR</i>	<u>k</u> aryogamy
<i>KEX</i>	<u>k</u> iller <u>e</u> xpression
<i>KRE</i>	<u>k</u> iller toxin <u>r</u> esistance
<i>SEC</i>	<u>s</u> ecretion
<i>SPC</i>	<u>s</u> ignal <u>p</u> eptidase <u>c</u> omplex (mammalian)
<i>SSR</i>	<u>s</u> ignal <u>s</u> equ <u>e</u> nce <u>r</u> eceptor (mammalian)
<i>SSS</i>	<u>s</u> ec <u>s</u> ixty-one <u>s</u> uppressor
<i>UBC</i>	<u>u</u> biquitin <u>c</u> onjugating enzyme
<i>WBP</i>	<u>w</u> heat germ agglutinin <u>b</u> inding <u>p</u> rotein

## ABSTRACTS

### Summary of the thesis

Membrane anchoring of cell surface proteins by means of glycosyl phosphatidyl-inositol (GPI) anchors is ubiquitous among eucaryotes. GPI anchoring is essential for yeast viability. In mammals, GPI anchoring is not required at the cellular level, but plays an important role in cell to cell and cell to environment interactions which for example are critical during embryogenesis. GPI proteins can have a wide variety of different functions, e.g. as immunoprotective parasitic surface coat proteins, yeast cell wall proteins, mammalian receptors, cell adhesion molecules, differentiation antigens and enzymes. Although several functions have been proposed for the GPI anchor itself, e.g. intracellular sorting, transmembrane signalling, or potocytosis, its most fundamental function consists in the attachment of proteins to the outer leaflet of the plasma membrane in an efficient and stable manner, comparable with a transmembrane polypeptide domain. However, GPI protein anchors may differ from transmembrane anchors in several respects such as lateral mobility, sensitivity to extracellular lipases, physical isolation from the cytoplasm and the cytoskeleton, and intercellular protein transfer. A GPI precursor protein is synthesized with a cleavable N terminal signal sequence required for the entry into the secretory pathway and a C terminal GPI signal sequence (GPI-SS) required for GPI anchor attachment. The process of GPI anchor attachment occurs in the lumen of the endoplasmic reticulum (ER) and consists in the transfer of a preformed GPI glycolipid onto an immature GPI protein which results in removal of the GPI-SS. The process was suggested to be catalyzed by a GPI:protein transamidase complex. Two putative subunits of this complex were identified, Gaa1p and Gpi8p. The exact role of Gaa1p is unknown, whereas Gpi8p, because of its homology to plant cysteine proteases, is believed to be the catalytic subunit of the transamidase complex. This study had as major objective the further characterization of the yeast Gpi8p itself and of its role in the process of GPI anchor attachment. By site-directed mutagenesis, the active site of Gpi8p consisting of a diad (C199 and H157) was identified and found to be similar to that of caspases. This supports the hypothesis that Gpi8p protein might be directly involved in removal of the C terminal GPI signal sequence. However, no Gpi8p mutant could be isolated in a screen with random PCR mutagenized *gpi8* alleles which shows proteolytic activity *in vivo*. Furthermore, a recently made hypothesis which claimed that human Gpi8p alone was responsible for the recognition of the GPI-SS of human placental alkaline phosphatase could be shown to be incorrect. This finding together with an experiment which showed that overexpression of Gpi8p could not suppress the partial maturation defect of Gas1p mutants having an altered GPI-SS, suggests that other proteins are involved in the specific recognition of the GPI-SS. A multicopy suppressor screen for genes which restored growth in wt cells overexpressing the dominant-negative active site mutant of Gpi8p, C199A, identified unknown genes which should be further analyzed. Moreover, two *gpi8* alleles were identified which show a strong temperature-sensitive defect for growth as promising tools for a synthetic lethality screen. A relational database for yeast GPI proteins was established. Furthermore, several ORFs were identified as candidates for enzymes which exchange the lipid moieties of GPI anchors by homology searches with www-based sequence analysis software. Their participation in the so-called lipid remodeling is currently tested.

## Zusammenfassung der Doktorarbeit

Die Verankerung von Zelloberflächenproteinen in der Zellmembran mit Hilfe von Glykosylphosphatidylinositol(GPI)-Ankern ist ubiquitär bei Eukaryonten. Die GPI-Verankerung ist essentiell für das Überleben von Hefezellen. In Säugetieren hingegen ist sie nicht erforderlich für das Überleben der einzelnen Zelle, spielt aber eine wichtige Rolle bei Interaktionen von Zellen untereinander und mit ihrer Umgebung wie sie beispielsweise bei der Embryogenese wichtig sind. GPI-Proteine sind für eine Vielzahl von verschiedenen Funktionen verantwortlich, wie z.B. als immunprotektive Oberflächenmantelproteine, Zellwandproteine in der Hefe, Säugetier-Rezeptoren, Zelladhäsionsmolekülen, Differenzierungsantigene, und als Enzyme. Obwohl den GPI-Ankern verschiedene spezielle Funktionen zugeschrieben wurden, wie z.B. beim intrazellulären Sorting, der transmembranären Signalübertragung, und der Potozytose, so besteht doch ihre fundamentalste Funktion im Verankern von Proteinen in der äusseren Lipidschicht der Plasmamembran, und zwar auf eine stabile und effiziente Weise, vergleichbar mit einer transmembranären Polypeptidomäne. Trotzdem unterscheiden sich GPI-Anker von transmembranären Ankern in vielerlei Hinsicht, nämlich der lateralen Mobilität, der Empfindlichkeit gegenüber extrazellulären Lipasen, der physikalischen Abschirmung vom Zytosol und Zytoskelett, und dem interzellulären Proteintransfer. Ein Vorläufer eines GPI-Proteins wird synthetisiert mit einer N-terminalen Signalsequenz, die notwendig für den Eintritt in den sekretorischen Weg, und einer C-terminalen GPI-Signalsequenz (GPI-SS), die verantwortlich für die GPI-Verankerung ist. Der Prozess der GPI-Verankerung läuft im Lumen des endoplasmatischen Retikulums (ER) ab und besteht in der Übertragung eines vorgebildeten GPI-Glykolipids auf ein unreifes GPI protein, was zu dessen Verlust der GPI-SS führt. Diese Reaktion wird vermutlich durch einen GPI:Protein-Transamidase-Komplex katalysiert. Zwei mögliche Untereinheiten dieses Komplexes wurden identifiziert, Gaa1p und Gpi8p. Welche Rolle Gaa1p spielt, ist unklar, während Gpi8p vermutlich die katalytische Untereinheit der Transamidase-Komplexes bildet, dies aufgrund von Homologien zu pflanzlichen Cysteinproteasen. Die vorliegende Studie hat als Hauptziel die weitere Beschreibung des Hefeproteins Gpi8p und seiner Rolle im Prozess der GPI-Verankerung. Durch gerichtete Mutagenese wurde das aktive Zentrum von Gpi8p als Diade (aus C199 und H157) identifiziert, welches ähnlich ist zu demjenigen in Caspasen. Das unterstützt die Hypothese, dass Gpi8p direkt beim Abschneiden der GPI-SS beteiligt sein könnte. Wie dem auch sei, mit ungerichteter PCR-Mutagenese konnte keine Gpi8p-Mutante gefunden werden, die *in vivo* proteolytisch aktiv war. Des weiteren konnte eine kürzlich gemacht Hypothese, welche besagte, dass Gpi8p alleine für die Erkennung der GPI-SS der menschlichen alkalinen Phosphatase der Plazenta verantwortlich sei, als falsch aufgezeigt werden. Diese Studie zusammen mit einem Experiment, welches zeigte, dass die Überexpression von Gpi8p den teilweisen Reifungsdefekt von Gas1p-Mutanten mit einer veränderten GPI-SS nicht supprimieren konnte, zeigt auf, dass neben Gpi8p wahrscheinlich auch andere Proteine bei der spezifischen Erkennung der GPI-SS beteiligt sind. Mit einem Multicopy-Suppressor-Screen für Gene, welche das Wachstum von Wildtyp-Zellen, die die dominant-negative Gpi8p-Mutante C199A überexprimierten, wiederherstellten, wurden unbekannte Gene identifiziert, die noch genauer analysiert werden müssen. Des weiteren wurden zwei *gpi8* Allele mit einem starken temperaturabhängigen Wachstumsdefekt als vielversprechende Werkzeuge für einen Synthetic-Lethality-Screen identifiziert. Eine relationale Datenbank für Hefe-GPI-Proteine wurde aufgebaut. Des weiteren, wurden verschiedene ORFs als Kandidaten für Enzyme, welche Lipide auf den GPI-Ankern austauschen, durch die Suche nach Homologien mit Hilfe von web-basierten Sequenzanalyseprogrammen gefunden. Ihr Mitwirken beim sogenannten Lipid-Remodeling wird zur Zeit getestet.

## Résumé de la thèse

L'attachement des protéines à la surface cellulaire par l'intermédiaire d'ancres glycosyl phosphatidylinositol (GPI) est ubiquitaire chez les eucaryotes. L'ancrage GPI est indispensable à la viabilité des levures. Chez les mammifères, bien que dispensable pour la survie d'une cellule isolée, il joue un rôle primordial lors des interactions cellule-cellule ou cellule-environnement, ainsi, par exemple lors de l'embryogénèse. Les protéines ancrées GPI remplissent un grand nombre de fonctions. A la surface de certains parasites elles ont un rôle immunoprotecteur, elles entrent dans la composition de la paroi chez les levures, sont des récepteurs chez les mammifères, interviennent lors de l'adhésion cellulaire, remplissent des fonctions enzymatiques ou comme antigènes de différenciation. Bien que différents rôles aient été proposés pour les ancres GPI, dont: l'adressage des protéines, la signalisation transmembranaire, et la potocytose, leur principale fonction reste l'attachement des protéines à la surface externe de la membrane plasmique d'une manière aussi efficace et stable que pour les domaines transmembranaires polypeptidiques. Cependant les ancres GPI diffèrent des ancres polypeptidiques à plusieurs titres: la mobilité latérale, la sensibilité aux lipases extracellulaires, l'indépendance vis-à-vis du cytoplasme et du cytosquelette et le mode de transport intracellulaire différent. Le précurseur d'une protéine GPI est synthétisé avec 2 séquences signal; l'une, en N terminal, permet l'entrée de la protéine dans la voie de sécrétion; l'autre, en C terminal, constitue la séquence signal GPI (GPI-SS) nécessaire à l'attachement de l'ancre GPI. Le transfert de l'ancre GPI a lieu dans le lumen du réticulum endoplasmique et consiste en l'attachement d'un glycolipide GPI déjà prêt à une protéine GPI immature dont la séquence signal GPI est coupée à ce moment. Il a été proposé que ce processus soit catalysé par un complexe transamidase GPI:protéine. Deux sous-unités pouvant appartenir à ce complexe ont été identifiés: Gaa1p et Gpi8p. Le rôle exact de Gaa1p est inconnue, cependant que Gpi8p, du fait de son homologie avec des protéases à cystéine végétales, pourrait être la sous-unité catalytique d'un éventuel complexe transamidase. L'objectif principal de ce travail est de mieux caractériser la protéine Gpi8p chez la levure, et de mieux définir son rôle dans l'attachement de l'ancre GPI. Par mutagenèse dirigée, le site actif de Gpi8p, composé d'une diade (C199 et H157) a été identifié; celui-ci est similaire au site actif des caspases. Cela renforce l'hypothèse selon laquelle Gpi8p serait directement impliquée dans la coupure de la séquence signal GPI en C terminal. Cependant aucun mutant *gpi8* n'a pu être isolé par un crible de mutants *gpi8* obtenus par mutagenèse "au hasard" par PCR, et ayant une activité protéolytique *in vitro*. De plus, une hypothèse récente selon laquelle la Gpi8p humaine, seule, serait responsable de la reconnaissance de la phosphatase alcaline placentaire humaine peut être considérée comme fautive. Cette étude ainsi que le fait que la surexpression de Gpi8p ne peut restaurer une maturation normale chez des mutants Gas1p ayant une séquence signal modifiée, suggèrent que d'autres protéines sont impliquées dans la reconnaissance spécifique de GPI-SS. Un crible destiné à rechercher des gènes capables de restaurer la croissance de cellules sauvages surexprimant l'allèle dominant-négatif de Gpi8p, C199A, a été fait. Les gènes de fonction inconnue ainsi identifiés doivent être plus amplement analysés. De plus, deux allèles de *GPI8* dont la croissance s'arrête complètement à la température restrictive ont été identifiés et sont des outils prometteurs pour un crible de "létales synthétiques". Finalement, une base de données relationnelle a été établie et un certain nombre de gènes pouvant être impliqués dans le processus de remodeling de la partie lipidique de l'ancre GPI ont été retenus après une analyse des séquences de protéines à l'aide de logiciels disponibles sur internet.

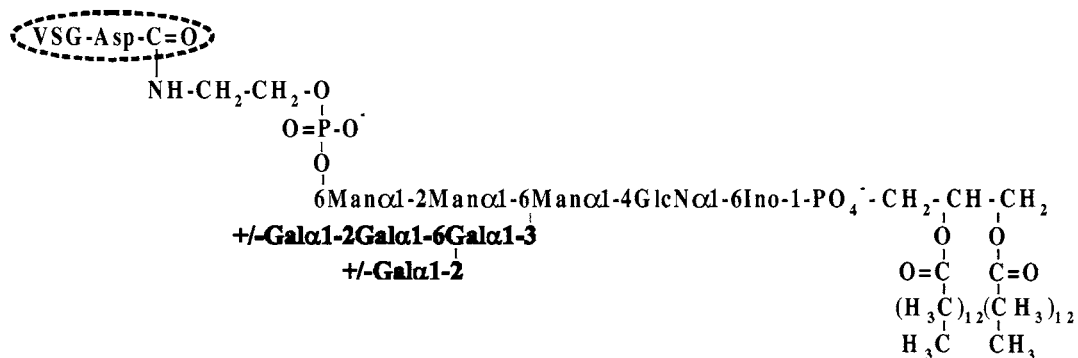
# INTRODUCTION

## 1.1. Discovery of GPI anchors and their occurrence

In eucaryotes, many glycoproteins are attached to the outer leaflet of the plasma membrane by means of a glycosylphosphatidylinositol (GPI) anchor covalently linked to the carboxy terminus.

The first indications for the existence of these lipid anchors came in the 1960s with the - at this time underestimated - observation that purified bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) could selectively release alkaline phosphatase from the surface of mammalian cells (Slein and Logan, 1965). In the late 1970s several studies could demonstrate that the alkaline phosphatase as well as other proteins, such as 5'-nucleotidase and acetylcholinesterase, can be released from plasma membranes by the action of highly purified bacterial PI-PLC (Ikezawa *et al.*, 1976; Low and Finean, 1977). In 1981 the first evidence for a covalent linkage between a phospholipid and a protein was provided in a study on the rat antigen Thy-1 of which the carboxy terminal proteolytic fragment showed stoichiometric amounts of ethanolamine and fatty acids (Campbell *et al.*, 1981).

A milestone in the history of GPIs was the discovery that the variant surface glycoprotein (VSG) isolated from the pathogen of the human sleeping sickness, *Trypanosoma brucei*, is anchored to the cell surface by a glycolipid containing phosphatidylinositol (Ferguson *et al.*, 1985). In contrast to mammalian GPI-anchored proteins being available only in small quantities, this protein is highly expressed in this parasite ( $10^7$  molecules per cell) and therefore easily purified in amounts necessary for the initial structural characterization of the GPI membrane anchor. The complete structure of the trypanosome anchor was published in 1988 (Ferguson *et al.*, 1988).



**Figure 1. The complete structure of the VSG GPI anchor from *Trypanosoma brucei*.**

The carboxy terminal asparatic acid residue of the VSG polypeptide is covalently linked via an amide bond with the ethanolaminephosphate group of the GPI anchor. The VSG GPI anchor has a backbone of ethanolaminephosphate-Man- $\alpha$ (1 $\rightarrow$ 2)-Man- $\alpha$ (1 $\rightarrow$ 6)-Man- $\alpha$ (1 $\rightarrow$ 4)-GlcN- $\alpha$ (1 $\rightarrow$ 6)-*myo*-inositolphosphate. Through the knowledge of an increasing number of GPI anchor structures from different sources, this GPI backbone (highlighted in light-blue) turned out to be conserved among eucaryotic GPI protein anchors. The proteinaceous part of the mature VSG protein is depicted in red, the lipid part and side chains are drawn in black and dark blue, respectively.

Since the basic methodological techniques to unravel the GPI anchor structure had been established, GPI anchors from a number of other proteins and from various eucaryotic organisms were structurally described in the following years (Tiede *et al.*, 1999; McConville and Ferguson, 1993). All these structures had a common property: the conserved GPI anchor backbone which can be embraced by different side chains and is linked to a variable lipid part, the composition of the latter two depending on the nature and the origin of the analyzed protein (Fig.1).

Several reviews and papers were published which collect knowledge on the structure, biosynthesis, processing and function of GPI anchors as well as of GPI proteins in lower and higher eucaryotes including yeast, slime mould, trypanosomes, fruit fly, worm, algae and higher terrestrial plants, birds, and mammals such as man, mouse and rat (Tiede *et al.*, 1999; Oxley and Bacic, 1999; Takos *et al.*, 1997; Morita *et al.*, 1996; Kinoshita *et al.*, 1997; Ferguson, 1999; Udenfriend and Kodukula, 1995; McConville and Ferguson, 1993). The presence of GPI-anchored proteins was also described in the archaeobacterium *Sulfolobus acidocaldarius* which is closely related to eucaryotes (Kobayashi *et al.*, 1997). Up to present, no evidence for the presence of GPI anchors in procaryotes was reported (Nosjean, 1998). However, GPI-like structures were described in several bacteria including *Pseudomonas syringae* and *Escherichia coli* (Jung *et al.*, 1998).

In summary, a consensus for the general structure and distribution of GPI membrane anchors emerged in 1985 and intensive biosynthetic, structural and functional studies started at the end of the eighties; a summary of the landmarks in GPI research has been published (Ferguson, 1999).

## 1.2. Biosynthesis of GPI glycolipid precursors

### 1.2.1. Biological membranes

Biological membranes consist mostly of lipids, proteins and mainly protein-bound carbohydrates. They form a complex and dynamic matrix of interactions which must be maintained when cell membranes expand, fuse, assemble and regenerate during active growth. In addition, a multiplicity of cellular phenomena such as membrane trafficking, signal transduction, compartmentalization, maintenance and establishment of gradients rely on the homeostasis of cellular membrane components. Therefore, it is not astonishing that general biochemical pathways for the biosynthesis and degradation of lipids have been preserved during the transition from procaryotic to eucaryotic organisms and are conserved among a variety of organisms ranging from yeast to mammals (Nikoloff and Henry, 1991; Carman and Zeimet, 1996).

Membrane lipids of *Saccharomyces cerevisiae* are similar to those of other eucaryotic organisms. Yeast cells are able to synthesize sterols, and its membranes typically contain a eucaryotic mixture of phospholipids. However, unlike higher eucaryotes, yeast cells do not synthesize ether lipids, and its fatty acids are predominantly 16 to 18 carbons in length. Lipid matrices of all subcellular membranes in yeast are constituted of essentially the same set of polar lipids and sterols. Nevertheless, the relative proportions vary considerably between different organelles and microdomains of organelles. Lipid compositions of defined membranes can be varied significantly by altering growth conditions such as

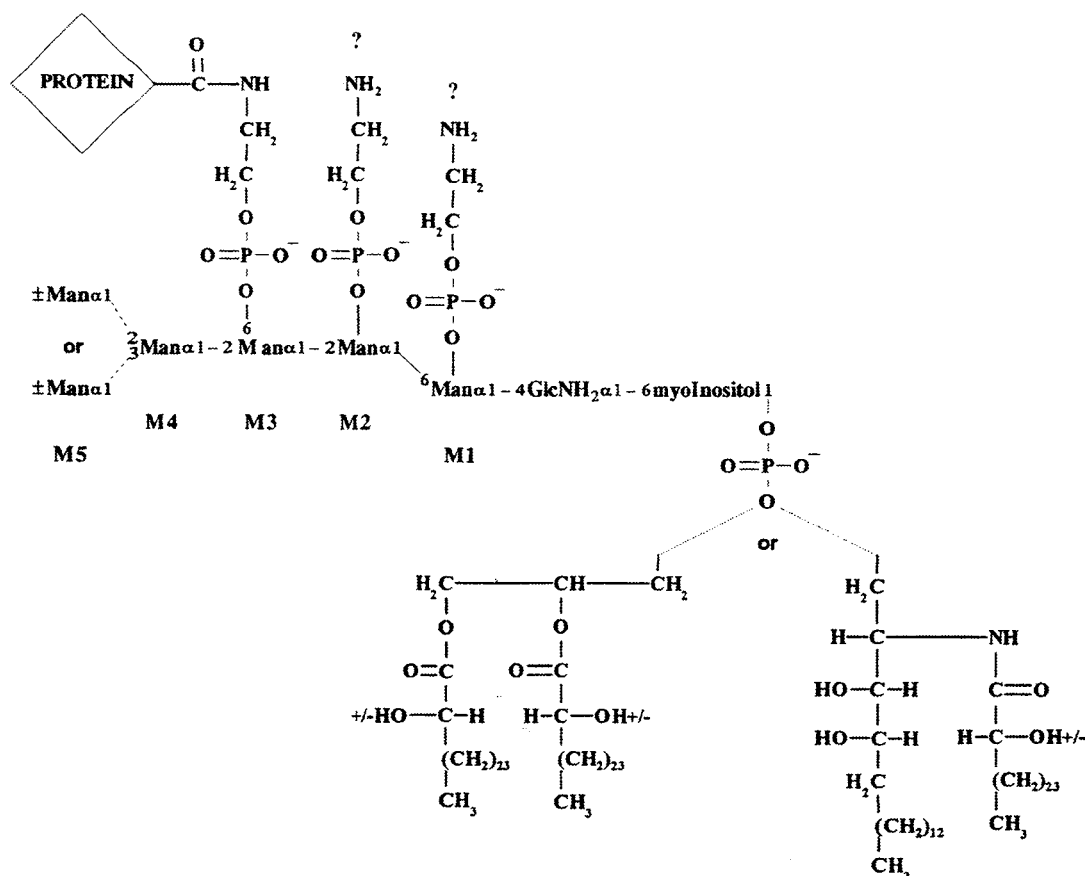
temperature, nutrient supply, oxygen concentration and growth rate (Henry, 1992). Differences in lipid composition between intracellular membranes cannot be adequately explained by local synthesis, modification and degradation (van Helvoort and van Meer, 1995). Lateral sorting of lipids in membranes, specificity in lipid transport by lipid transfer or vesicular transport, and lipid flippases, all play a role in intracellular lipid heterogeneity which is observed between different subcellular membranes, between inner and outer leaflets of the very same membrane as well as between different membrane microdomains. Different lipid transport pathways were discussed (van Helvoort and van Meer, 1995).

Eucaryotic membranes separate the cytosol from the external environment and from the lumen of the various organelles, whereby they compartmentalize the cellular metabolism. Membrane proteins are essential for maintaining the appropriate chemical conditions inside organelles, examples for such membrane proteins are channels, transporters, and proteins of the translocation machinery. Furthermore, membrane proteins play various roles in other important cellular mechanisms such as signal transduction, vesicular traffic, association of membrane microdomains, membrane coats, cell-to-cell interactions.

In addition, it becomes increasingly clear that membranes serve as scaffolds to organize chemical events in the cytosol. In certain cases, this is mediated in close interaction with the cytoskeleton (Berridge and Irvine, 1989; Nishizuka, 1992). Glycosylphosphatidylinositols (GPIs), either free or destined for transfer onto a newly synthesized GPI protein, are minor components of membranes. Typical for glycolipids, they are nearly exclusively located in the exoplasmic leaflet of membranes (lumenal side of organellar membranes or on the extracellular side of the plasma membrane). The structure and biosynthesis of complete GPI precursor glycolipids is summarized in the following.

### 1.2.2. Structure of GPI anchors

The structure of all protein-bound GPI anchors structurally explored so far consists of a conserved backbone ethanolaminephosphate-Man- $\alpha(1\rightarrow2)$ -Man- $\alpha(1\rightarrow6)$ -Man- $\alpha(1\rightarrow4)$ -GlcN- $\alpha(1\rightarrow6)$ -*myo*-inositolphosphate (Figure 1). Depending on the organism, the organ as well as on protein, their backbone may be additional groups decorated by different additional groups such as ethanolaminephosphate or ethanolaminephosphonate, galactose, N-acetylgalactosamine, mannose and N-acetylneuraminic acid (Fankhauser *et al.*, 1993; McConville and Ferguson, 1993; McConville *et al.*, 1993). Furthermore, the inositol ring can be acylated generally at an early step in GPI anchor biosynthesis (step 3; Table 1), and can be deacylated at later steps (Güther and Ferguson, 1995; Chen *et al.*, 1998). The lipid moiety attached via a phosphate group to the inositol moiety of the GPI backbone is also rather variable. Generally, glycerols such as *sn*-1-alkyl-2-acylglycerols, *sn*-1,2-diacylglycerols, *sn*-1-acyl-2-lyso-glycerols and *sn*-1-alkyl-2-lyso-glycerols are found (McConville and Ferguson, 1993; Bütikofer and Boschung, 1995), whereas in *Saccharomyces cerevisiae* and *Dictostelium discoideum*, ceramides were discovered as well (Fankhauser *et al.*, 1993; Stadler *et al.*, 1989; Haynes *et al.*, 1989). Figure 2 depicts the structures of GPI protein anchors in yeast. The structure of complete GPI precursor glycolipids were described (Sipos *et al.*, 1994).



**Figure 2. GPI anchor structures of yeast proteins.**

Most of the yeast proteins contain a ceramide as the lipid moiety. However, the lipid portion of the anchor of the abundant 125 kDa Gas1p consists of *lyso*- or diacylglycerol. The C26:0 fatty acid of both the diacylglycerol and the ceramide can be mono-hydroxylated. Mannoses are indicated by M1 to M5. In eighty percent of yeast GPI anchors only four mannose residues (M1 to M4) are present, whereas the other twenty percent of anchors contain an additional fifth mannose. There is no evidence for the presence of ethanolaminephosphate on M1 and M2 of GPI protein anchors (Fankhauser *et al.*, 1993), however, it has been suggested that they are added during GPI biosynthesis and are critical for the proper function of GPI proteins (Benachour *et al.*, 1999; Canivenc *et al.*, 1998).



### 1.2.3. GPI anchor biosynthesis

Because of the apparent conservation of the GPI backbone, one can expect that the basic biosynthetic pathway for GPIs is also conserved among eucaryotic organisms. This is in fact the case although interesting differences between protozoan, yeast and mammalian GPI biosynthetic pathways exist, mainly in terms of the modification of the GPI backbone. Here GPI biosynthesis in yeast and mammals are described side-by-side with some comments on the system in parasites; all systems were intensively reviewed (Masterson *et al.*, 1990; Thomas *et al.*, 1990; McConville and Ferguson, 1993; Herscovics and Orlean, 1993; Englund, 1993; Yeh *et al.*, 1994; Takeda and Kinoshita, 1995; Kinoshita *et al.*, 1997; Tiede *et al.*, 1998). Genes required for GPI anchor biosynthesis are spread over the entire genome in all organisms analyzed so far and do not form gene clusters. In Table 1 yeast and mammalian genes required for GPI biosynthesis are listed together with the mutants leading to the identification of many of the corresponding genes and with the orthologs in the other organism, if discovered. Figure 3 demonstrates a general overview on GPI anchoring in mammals.

#### *Steps in GPI protein anchor biosynthesis*

##### **1. Synthesis of N-acetylglucosaminyl phosphatidylinositol**

The first step in GPI anchor biosynthesis is the transfer of GlcNAc from UDP to PI to form the first GPI intermediate, GlcNAc-PI. The reaction is catalyzed by an  $\alpha$ 1-6 GlcNAc transferase found to be an ER complex of at least 4 subunits encoded by the mammalian genes *PIG-A*, *PIG-H*, *PIG-C* and *GPII* (Watanabe *et al.*, 1998). In yeast three genes, *GPII*, *GPI2*, and *GPI3/SPT14/CWH6* were shown to be required for this step. No homolog for the mammalian Pig-H was discovered so far in yeast. The quaternary complex was partially purified by affinity chromatography and was capable of *in vitro* GlcNAc-PI biosynthesis (Watanabe *et al.*, 1998). However, the exact role of the four subunits of this complex remains unclear. Because of its homology to a bacterial GlcNAc transferase, Pig-a was suggested to be the catalytic subunit of this complex (Bessler *et al.*, 1994; Kawagoe *et al.*, 1994). Furthermore, an active cysteine residue has been implied for Pig-a since it was reported in a trypanosome cell-free system that inhibition of GlcNAc transferase activity by sulfhydryl alkylating reagents can be prevented by prior addition of UDP-GlcNAc (Milne *et al.*, 1992).

For Gpilp, some functional insight came from *S. cerevisiae* and *S. pombe*  $\Delta$ gpi1 cells which were complemented by mammalian Gpilp (Tiede *et al.*, 1998).  $\Delta$ gpi1 of *S. pombe* is lethal and growth could be restored by hGpilp.  $\Delta$ gpi1 of *S. cerevisiae* is viable but shows a temperature-sensitive (ts) phenotype for growth (Leidich and Orlean, 1996a). GlcNAc transferase activity present *in vivo* could not be detected *in vitro* but was increased by expression of hGpilp. However, the mutant remained temperature-sensitive suggesting that either the hGpilp itself or the complex was labile (Tiede *et al.*, 1998). In addition, overexpression of Gpi2p could partially suppress the ts phenotype of the gpi1 deletant indicating that its function can be bypassed (Leidich *et al.*, 1995a). The current view on the function of Gpilp suggests that Gpilp might be involved in assembly and/or stabilization of the GlcNAc-PI biosynthetic complex, which was supported by Watanabe *et al.* (1998) who showed

that Gpilp interacts with all three other subunits of the complex. A further study which reported a drastic decrease in the amount of the complex containing Pig-a, Pig-c and Pig-h if Gpilp was depleted provided further evidence (Hong *et al.*, 1999a). Nonetheless, Gpilp might also have some regulatory functions since a putative phosphorylation site is conserved in all Gpilp homologs.

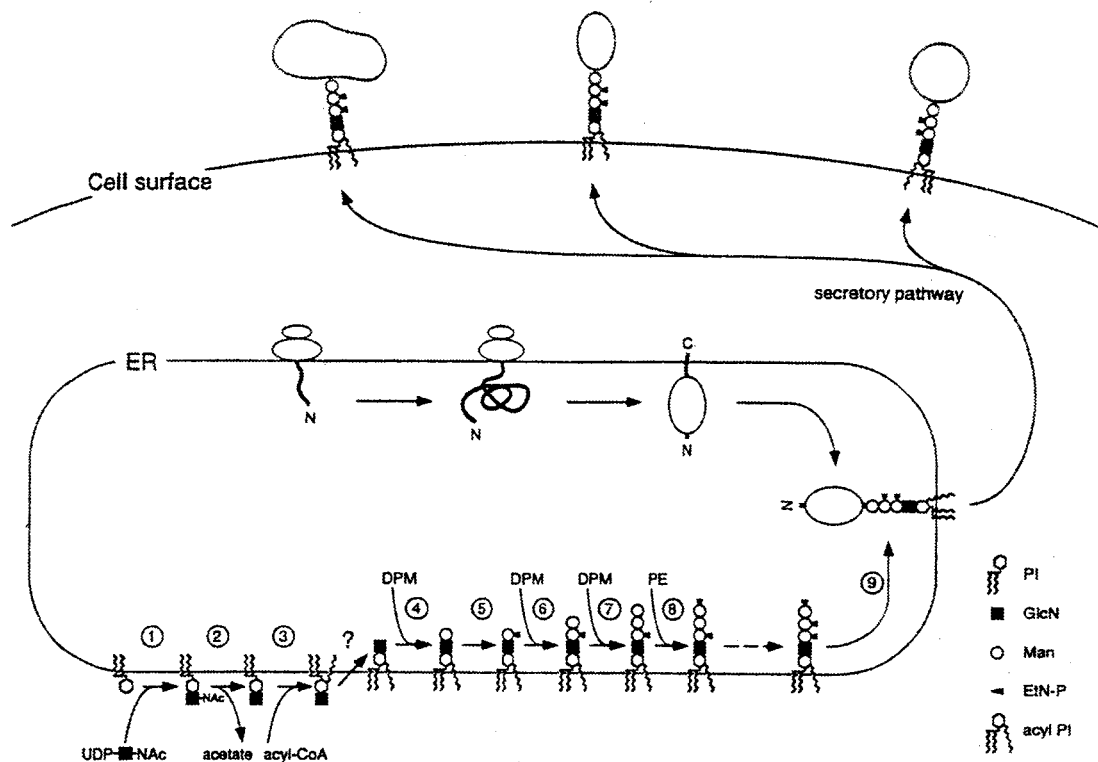
The roles for Pig-c/Gpi2 and Pig-h remain unclear. Nevertheless, since the biosynthetic defect of class H mutants could be bypassed by the addition of sodium butyrate (Tisdale *et al.*, 1991), Pig-h was hypothesized to be an activator or regulator of GlcNAc-PI biosynthesis, a function not present in yeast cells and being a unique feature of mammalian cells (Mensa-Wilmot *et al.*, 1994).

## 2. De-N-acetylation of GlcNAc-PI

The second step consists of deacetylation of GlcNAc-PI to form GlcN-PI. No deacetylase activity could be found in the complex required for the first step indicating that first and second step of GPI biosynthesis are locally separated (Nakamura *et al.*, 1997). A rat cDNA which complemented deacetylase deficiency of a CHO class L mutant was isolated by expression cloning (Nakamura *et al.*, 1997). The yeast homolog of the mammalian *PIG-L* was then discovered by homology searches and named *GPI12*. Both were shown to be *N*-acetylglucosaminylphosphatidylinositol de-N-acetylases and to be essential for GPI anchoring (Watanabe *et al.*, 1999). The Pig-l protein is a type II membrane protein, most of it residing on the cytosolic face of the ER.

## 3. Reversible inositol acylation

During the third step, GlcN-PI is acylated on the inositol ring to form GlcN-(acyl)PI. Generally, a palmitoyl group is transferred by a not yet identified enzyme. In contrast to trypanosomes, inositol acylation is an obligatory step in both mammals and yeast (Herscovics and Orlean, 1993; Doerrler *et al.*, 1996; Guthrie 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 proteins analyzed in this way were PI-PLC sensitive indicating that this acylation is reversible (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).



**Figure 3. Biosynthesis of GPI-anchored proteins in the mammalian system.**

Biosynthesis of both the GPI anchor and the GPI protein precursor are depicted schematically. Numbering of GPI biosynthetic steps coincide with the reaction steps described in the text. Definite evidence for the precise sequence of reactions was published up to step 4, the sequence of the following reaction was discussed (Flury *et al.*, 2000; Tiede *et al.*, 1999; Kinoshita *et al.*, 1997). After GPI anchor addition, the proteins are transported through the secretory pathway to the plasma membrane (mammals, yeast) and incorporated into the cell wall of yeast cells (not depicted). GPI anchoring of yeast and mammals are very similar, few differences are mentioned in the text. (Kinoshita *et al.*, 1997)

#### 4. Addition of the first mannose

Mannose is transferred from dolicholphosphomannose (Dol-P-Man) to GlcN-acylPI which generates Man-GlcN-acylPI. The putative GlcN-acylPI mannosyltransferase I (GPI-MT-I) has not been identified so far. However, GPI-MT-I activities in trypanosomes compared to the mammalian and yeast system appear to differ in terms of specificity since inositol acylation is not a prerequisite for mannosylation in the parasite system. In the mammalian cell-free system, transfer of the first mannose residue requires inositol acylation of GlcN-PI (Doerrler *et al.*, 1996; Smith *et al.*, 1997). Therefore, the mammalian pathway proceeds in the order: 1. de-*N*-acetylation, 2. inositol acylation, and 3. transfer of the first mannose. In the trypanosome cell-free system, inositol-acylated GPI intermediates are found starting from Man-GlcN-PI onwards indicating that the reaction order is: 1. de-*N*-acetylation, 2. transfer of the first mannose, and 3. inositol acylation. It has been suggested that in contrast to the mammalian pathway, substrate channeling from the de-*N*-acetylase to the GPI-MT-I occurs prior to inositol acylation and mediates biosynthesis of Man-GlcN-PI since in an trypanosome cell-free system, GlcN-PI entered the pathway less efficiently than GlcNAc-PI (Smith *et al.*, 1996). This in turn could explain why GlcN-PI is not inositol-acylated prior to the addition of the first mannose (Tiede *et al.*, 1999).

### 5. Transfer of ethanolaminephosphate onto the first mannose

In yeast and in mammalian cells, a first ethanolaminephosphate (EtN-P) residue is added to position 2 of the mannose residue most probably at the stage of Man-Glc-(acyl)PI resulting in (EtN-P)Man-GlcN-(acyl)PI (Homans *et al.*, 1988; Puoti *et al.*, 1991; Puoti and Conzelmann, 1993). In yeast, the donor of EtN-P was shown to be phosphatidylethanolamine (Imhof *et al.*, in press). The yeast ethanolaminephosphate transferase responsible for this step in yeast was recently discovered by cloning a mutant defective in bud emergence and named Mcd4p (Gaynor *et al.*, 1999). Mcd4 function is essential for yeast cell viability, encodes a multimembrane-spanning protein that localizes to the ER and contains a large N terminal ER lumenal domain (Gaynor *et al.*, 1999). Human Mcd4p was isolated by homology searches with yeast Mcd4p; Mcd4 is highly conserved among eucaryotes sequenced so far and has two yeast homologs which were suggested to be responsible for ethanolaminephosphate transfer onto the second and third mannose, being Gpi7p and Gpi13p/Yli031p (Benachour *et al.*, 1999; Flury *et al.*, 2000; Taron *et al.*, 2000).

### 6. Addition of the second mannose

Another second, still unknown Dol-P-Man:(EtN-P)Man-GlcN-(acyl)PI mannosyl-transferase, GPI-MT-II, is responsible for the transfer of a second mannose residue which results in Man-(EtN-P)Man-GlcN-(acyl)PI.

### 7. Addition of the third mannose

The seventh step of GPI protein anchor biosynthesis consists of the transfer of a third mannose from Dol-P-Man onto the Man-(EtN-P)Man-GlcN-(acyl)PI (M2) intermediate forming Man-Man-(EtN-P)Man-GlcN-(acyl)PI. A possible candidate for this putative GPI-MT-III has been identified by complementing a mammalian mutant cell line of complementation class B which lead also to the identification of the yeast gene *GPI10* being defective in the yeast mutant 839 (Sugiyama *et al.*, 1991; Puoti and Conzelmann, 1993; Canivenc-Gansel *et al.*, 1998a; Sütterlin *et al.*, 1998). Both the mammalian and the yeast mutant accumulate an M2 precursor. The structure of the intermediate accumulated by outcrossed 839 mutant *gpi10-1* revealed the structure Man-(EtN-P)Man-GlcN-(acyl)PI. This structural data combined with the data on ethanolaminephosphate transferases in yeast suggest that the ethanolaminephosphate on the second mannose is transferred most probably at a later step occurring after the transfer of a third mannose (Flury *et al.*, 2000).

Pig-b protein encodes an ER membrane protein with a large intraluminal C-terminus and a small cytoplasmic N-terminus that is not necessary for function (Takahashi *et al.*, 1996). Beyond yeast Gpi10p, Pig-b shows homology to three additional yeast proteins: Smp3 protein which may to add the fourth mannose to Man-Man-(EtN-P)Man-GlcN-(acyl)PI, and *Alg9* and *YNR030w* which are non-essential genes involved in *N*-linked glycosylation (Tiede *et al.*, 1999 and references therein). It remains to be demonstrated whether the Pig-b/Gpi10p is the complete GPI-MT-III or whether it acts as a subunit of a heterooligomeric complex.

### 8. Transfer of ethanolaminephosphate onto the third mannose

Step 8 defines the transfer of EtN-P onto position 6 of the third mannose, a step which is supposed to be preceded by the addition of a fourth mannose in yeast (Flury *et al.*, 2000). The corresponding EtN-P transferase has been recently identified in yeast as

an essential protein homologous to Gpi7p and Mcd4p being named Gpi13p/YII031c (Flury *et al.*, 2000; Taron *et al.*, 2000). Gpi13p-deficient cells accumulate a GPIs lipid with the structure containing Man<sub>4</sub>-GlcN-(acyl)PI with or without EtN-P on the first mannose (Flury *et al.*, 2000; Taron *et al.*, 2000). The human homolog of Gpi13p, Pig-o was also cloned (Hong *et al.*, 2000).

In mammals, Pig-F, a very hydrophobic ER protein of 219 amino acids cloned by expression cloning of a Class F mutant defective in this step (Sugiyama *et al.*, 1991; Puoti and Conzelmann, 1993) was implied in the transfer of EtN-P onto the third mannose as well (Inoue *et al.*, 1993). The role of Pig-f and of its yeast homolog, Gpil1p, is unclear. GPI anchoring and formation of complete GPI precursors is blocked in *gpi11* disruptants which can be complemented by the human Pig-f although complemented cells are temperature-sensitive for growth and accumulate two types of GPIs, a less polar GPI with EtN-P on the second mannose and a more polar GPI with EtN-P on the third mannose and probably another EtN-P on the second or first mannose. The lipid profile of  $\Delta gpi11$  resembles that of cells lacking Gpi7p which is suggested to add EtN-P onto the second mannose. Therefore, Gpil1p was suggested to not affect EtN-P transfer onto the third but rather on the second mannose; possible roles of Gpil1p in the supply of EthN-P were discussed (Taron *et al.*, 2000).

#### **Transfer of ethanolaminephosphate onto the second mannose**

A further EtN-P residue can be present at position 6 of the second mannose residue (Deeg *et al.*, 1992; Taron *et al.*, 2000). Transfer of ethanolaminephosphate onto the second mannose at a later step has been suggested to be catalyzed by the Gpi7p isolated in a screen for yeast mutants unable to present the GPI cell wall protein  $\alpha$ -agglutinin on their surface (Benghezal *et al.*, 1995; Benachour *et al.*, 1999; Flury *et al.*, 2000). 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. Several aspects of GPI biosynthesis are disturbed in  $\Delta gpi7$ . The extent of anchor remodeling, i.e. replacement of the primary lipid moiety of GPI anchors by ceramide, in the Golgi is significantly reduced, and the transport of GPI proteins to the Golgi is delayed. Furthermore,  $\Delta gpi7$  accumulates a GPI lipid lacking a side chain on the second mannose. It is still not known how Gpi7p is involved in GPI anchor biosynthesis since the bulk of the protein has been localized to the plasma membrane (Benachour *et al.*, 1999).

Protozoal GPI intermediates do not contain EtN-P on the first or second mannose.

#### **Synthesis of dolicholphosphate mannose**

DPM is the mannose donor for all GPI-MT enzymes. DPM plays not only a role in GPI anchor biosynthesis but also in the assembly of the precursor oligosaccharide for *N*-glycosylation of eucaryotic glycoproteins and in fungi, also for *O*-mannosylation (Kornfeld and Kornfeld, 1985; Herscovics and Orlean, 1993). DPM is synthesized from GDP-mannose and dolicholphosphate by DPM synthase in the endoplasmic reticulum. DPM synthases were described in various organisms and were grouped into two types: one type is a single enzyme represented e.g. by *S. cerevisiae* Dpm1p (Colussi *et al.*, 1997), a second type is a multi-component enzyme represented by human DPM synthase, recently reported to consist of three subunits Dpm1p, Dpm2p, and Dpm3p, the first being the catalytic subunit (Maeda *et al.*, 2000).

## 9. GPI anchor attachment

The last step of GPI anchor biosynthesis comprises the addition of a complete GPI precursor onto a GPI precursor protein, the biosynthesis of the latter is described in the following section.

Including the data of *Δgpi7* cells, it is presumed that in yeast both structures, Man-(EtN-P)Man-Man-(EtN-P)Man-GlcN-(acyl)PI and the Man-(EtN-P)Man-(EtN-P)Man-(EtN-P)Man-GlcN-(acyl)PI, the latter structure named complete GPI precursor CP2 (Benachour *et al.*, 1999), can be transferred onto GPI precursor proteins. The same structures but lacking the fourth mannose residue were described as mature GPI anchor precursors in the mammalian system (Hirose *et al.*, 1995; Yeh *et al.*, 1994; Puoti *et al.*, 1992). A second complete precursor glycolipid, CP1, predominating in yeast cells grown at 24 °C was reported to differ from CP2 with regard to the fatty acids of their diacylglycerol moiety (Sipos *et al.*, 1994).

**Table 1. GPI biosynthetic genes of yeast and mammalian cells.**

Step	Function	Yeast			
		mutants	gene	mammalian homolog	references
1	<b>Transfer of GlcNAc onto PI</b>	<i>spt14</i>	<i>SPT14</i>	<i>PIG-A</i>	(Schonbachler <i>et al.</i> , 1995)
		<i>gpi3</i>			(Leidich <i>et al.</i> , 1995b)
		<i>cwh6</i>			(Vossen <i>et al.</i> , 1995; Vossen <i>et al.</i> , 1997)
		<i>gpi1</i>	<i>GPI1</i>	<i>hGPI1</i>	(Leidich and Orlean, 1996b)
		<i>gpi2</i>	<i>GPI2</i>	<i>PIG-C</i>	(Leidich <i>et al.</i> , 1995b)
2	<b>GlcNAc-PI-de-N-acetylation</b>		<i>GPI12</i>	<i>PIG-L</i>	(Watanabe <i>et al.</i> , 1999)
3	<b>GlcNAc-PI acylation/deacylation</b>				
4	<b>GlcN-(acyl)PI mannosylation (synthesis of M1)</b>				
5	<b>Transfer of EtN-P onto M1</b>	<i>mcd4</i>	<i>MCD4</i>	<i>PIG-N</i>	(Gaynor <i>et al.</i> , 1999)
6	<b>Mannosylation of mannosyl-GlcN-(acyl)PI (synthesis of M2)</b>				
7	<b>Mannosylation of dimannosyl-GlcN-(acyl)PI (synthesis of M3)</b>	839	<i>GPI10/YGL142c</i>	<i>PIG-B</i>	(Benghezal <i>et al.</i> , 1995) (Sütterlin <i>et al.</i> , 1998) (Canivenc-Gansel <i>et al.</i> , 1998b)
8	<b>Transfer of EtN-P onto M3</b>		<i>GPI13/YLL031c</i>	<i>PIG-O</i>	(Taron <i>et al.</i> , 2000) (Flury <i>et al.</i> , 2000)
	<b>Transfer of EtN-P onto M2</b>	<i>gpi7</i>	<i>GPI7</i>	<i>n.k.*</i>	(Benachour <i>et al.</i> , 1999)
			<i>GPI11</i>	<i>PIG-F</i>	(Taron <i>et al.</i> , 2000)

	Synthesis of dolichol phosphate mannose	<i>dpm1</i>	<i>DPM1</i>	<i>DPM1</i>	(Orlean <i>et al.</i> , 1988)
9	GPI anchor attachment	<i>gaa1/ end2</i>	<i>GAA1</i>	<i>GAA1</i>	(Hamburger <i>et al.</i> , 1995)
		<i>gpi8</i>	<i>GPI8</i>	<i>GPI8</i>	(Benghezal <i>et al.</i> , 1996)
Step	Function	Mammalian cells			
		mutant	gene	yeast homolog	reference
1	Transfer of GlcNAc onto PI	Class A	<i>PIG-A</i>	<i>SPT14</i>	(Miyata <i>et al.</i> , 1993)
		Class C	<i>PIG-C</i>	<i>GPI2</i>	(Inoue <i>et al.</i> , 1996)
		Class H	<i>PIG-H</i>	n.k.*	(Kamitani <i>et al.</i> , 1993)
			<i>hGPI1</i>	<i>GPI1</i>	(Watanabe <i>et al.</i> , 1998)
2	GlcNAc-PI-de-N-acetylation		<i>rPIG-L</i>	<i>GPI12</i>	(Nakamura <i>et al.</i> , 1997)
		Class J			(Watanabe <i>et al.</i> , 1999)
					(Mohney <i>et al.</i> , 1994a)
					(Stevens <i>et al.</i> , 1996)
3	GlcNAc-PI acylation/deacylation				
4	GlcN-(acyl)PI mannosylation (synthesis of M1)				
5	Transfer of EtN-P onto M1		<i>PIG-n</i>	<i>MCD4</i>	(Hong <i>et al.</i> , 1999b)
6	Mannosylation of mannosyl-GlcN-(acyl)PI (synthesis of M2)				
7	Mannosylation of dimannosyl-GlcN-(acyl)PI (synthesis of M3)	Class B	<i>PIG-B</i>	<i>GPI10</i>	(Takahashi <i>et al.</i> , 1996)
8	Transfer of EtN-P onto M3		<i>PIG-O</i>	<i>GPI13</i>	(Hong <i>et al.</i> , 2000)
		Class F	<i>PIG-F</i>	<i>GPI11</i>	(Inoue <i>et al.</i> , 1993)
					(Ohishi <i>et al.</i> , 1996)
	Transfer of EtN-P onto M2		n.k.*	<i>GPI7</i>	
	Synthesis of dolichol phosphate mannose	Class E	<i>DPM1/ PIG-E</i>	<i>DPM1</i>	(Tomita <i>et al.</i> , 1998)
			<i>DPM2</i>		(Maeda <i>et al.</i> , 1998)
			<i>DPM3</i>		(Maeda <i>et al.</i> , 2000)
9	GPI anchor attachment		<i>GAA1</i>	<i>GAA1</i>	(Hiroi <i>et al.</i> , 1998a; Hiroi <i>et al.</i> , 1998b)
			<i>GPI8</i>	<i>GPI8</i>	(Yu <i>et al.</i> , 1997)

\* not known

### 1.3. Biosynthesis of GPI precursor proteins

#### 1.3.1. Yeast Genome and Proteome

The complete genome of yeast *Saccharomyces cerevisiae* was sequenced through a worldwide collaboration and released as first complete eucaryotic organism in 1996 (Goffeau *et al.*, 1996). The yeast proteome has been predicted to embrace approximately 5885 potential proteins of which 70 were predicted and 50 experimentally shown to be GPI-anchored (chapter 3.6.2.); this represents 0.85 to 1.2% of the total yeast genome. These numbers are clearly higher than that calculated for the proteome of *C. elegans*. In *C. elegans*, 86 GPI proteins were predicted which amounts to 0.45 % of the total number of proteins (Eisenhaber *et al.*, 2000). No automatic annotation of GPI proteins was reported in other organisms up to date.

#### 1.3.2. Biosynthesis of GPI proteins

Most cellular proteins are synthesized in the cytoplasm, however, some reside in membrane-bound organelles or outside of the cell. The subcellular localization of all mature GPI proteins identified so far is either the plasma membrane, the extracellular medium after cleavage of the formerly attached GPI anchor or for certain yeast proteins, the cell wall. This implies that GPI proteins have to enter the secretory pathway before reaching their final destination, as typical secretory proteins do.

##### Entry into the secretory pathway

Secretory protein biogenesis begins with the insertion of a preprotein, which contains at its N terminus a signal peptide for ER targeting, into the lumen of the endoplasmic reticulum (ER). In yeast, as in other eucaryotic cells, ER protein translocation, can occur either posttranslationally, in which the preprotein is completely synthesized on cytosolic ribosomes before being translocated, or cotranslationally, in which membrane-associated ribosomes direct the nascent polypeptide chain into the ER concomitant with polypeptide elongation (Brodsky, 1998; Panzner *et al.*, 1995). Depending on the hydrophobic core of the signal sequence, the transported polypeptide is translocated either cotranslationally, posttranslationally or by both pathways (Ng *et al.*, 1996).

In all cases, the preproteins are targeted to the ER membrane through specific interactions with cytosolic and/or ER membrane factors. Cotranslational translocation for example is initiated by binding of a ribosome-bound nascent polypeptide chain to SRP which subsequently binds to its membrane receptor (Walter and Johnson, 1994). SRP cycles on and off translating ribosomes at specific steps during the elongation cycle to inspect all nascent chains for the presence of signal sequences (Ogg and Walter, 1995).

The preprotein is then transferred to a multiprotein translocation machine in the ER membrane that includes a pore through which the preprotein passes into the ER lumen (Matlack *et al.*, 1998). The protein-conducting channels in the ER membrane of yeast and mammalian cells are formed by the heterotrimeric Sec61 complex comprising Sec61p, Sbh1p and Sss1p (in mammals Sec61 $\alpha$ , Sec61 $\beta$  and Sec61 $\gamma$ , respectively). A non-essential homolog of Sec61p, Ssh1p, forms a complex homologous to the Sec61



complex that contains Sbh2p and Sss1p; this complex has a special role for cotranslational translocation in yeast and has not been identified in the mammalian system up to now (Finke *et al.*, 1996). For posttranslational translocation, the tetrameric Sec63 complex (Sec63p, Sec62p, Sec71p and Sec72p) is required in addition to the Sec61 complex, both together forming the heptameric Sec complex (Panzner *et al.*, 1995).

Several models were established to explain how transport across membranes might be driven: 1. a brownian motion/molecular ratchet that traps the polypeptide and might depend on luminal chaperones, 2. an import motor that pulls the polypeptide, 3. or a combination of 1. and 2. (Römisch., 1999; Lyman and Schekman, 1997).

The energy required to drive protein translocation has been suggested to derive either from the coupling of translation to translocation (during cotranslational translocation) or from ER luminal molecular chaperones such as BiP that may harness the preprotein or regulate the translocation machinery (during post-translational translocation).

### **The GPI protein precursor: structure and maturation**

An immature GPI protein destined for modification by a preformed GPI anchor is synthesized with a cleavable N terminal signal sequence and a C terminal GPI signal sequence (GPI-SS) for GPI anchor attachment (Udenfriend and Kodukula, 1995). The N terminal signal sequence targets the growing polypeptide to the endoplasmic reticulum where it is translocated across and inserted into the ER membrane by both signal sequences. In the following, the N terminal signal sequence is cleaved off by the signal peptidase, and the C terminal GPI-SS is recognized by the ER luminal GPI:protein transferase which is suggested to act as a transamidase, i.e. to simultaneously remove the part of the GPI-SS located downstream of the GPI anchor attachment site and to replace it with a preformed GPI precursor glycolipid (cf. following section). However, it remains unclear in which precise sequence of actions the two signal sequences are recognized and processed.

### **1.3.3. Degradation of immature GPI proteins**

Eucaryotic cells have developed different mechanisms to ensure that the products of biosynthesis are accurate. For secretory proteins such quality control is frequently mediated by selective intracellular retention and degradation (Kopito, 1997). Misfolded secretory proteins are not degraded in the ER lumen, but are exported through the Sec61p channel by a retrograde translocation mechanism and then degraded by proteasomes present in the cytosol or in close neighbourhood to the cytosolic side of the ER membrane, a process called ER degradation. Factors required for protein export to the cytosol were discussed (Römisch., 1999). Within the last three or four years, knowledge on the degradation of unfolded secretory proteins, the ER degradation pathway and its components increased enormously (Hill and Cooper, 2000; Plempler *et al.*, 1999; Riezman, 1997). However, less is known about the fate of immature GPI proteins which were not modified by a GPI anchor. An uncleaved GPI-SS appears to act as a determinant for ER retention. Gas1p with a mutated GPI-SS was reported to be retained in the ER (Nuoffer *et al.*, 1993; Hamburger *et al.*, 1995). However, it is not clear how this retention is mediated: retention of unprocessed GPI proteins might be due to an active retention mechanism for which the

uncleaved GPI-SS is responsible, or might simply be due to the lack of an obligatory forward signal which may reside in the GPI anchor. Furthermore, failure to complete proteolytic cleavage of and anchor addition to a hGH/DAF fusion protein resulted in retention of the uncleaved GPI protein precursor in a post-ER compartment and in degradation by an ER degradation mechanism or a process resembling autophagy (Field *et al.*, 1994). Of those, autophagy was excluded since another study showed that degradation of hGH/DAF with an uncleavable GPI-SS was insensitive to treatment with nocodazole or compounds preventing cytoplasmic autophagy (Wainwright and Field, 1997). Recent evidence with the same mutant fusion protein showed that MG-132, a potent inhibitor of the proteasomes, partially protected mutant hGH/DAF from degradation suggesting that cytosolic proteasomes are involved (Wilbourn *et al.*, 1998). Further analysis has to be performed in order to understand whether besides the classical ER degradation pathway leading to cytosolic proteasomes, there are other components of the ER degradation pathway involved in degradation of immature, ER-retained GPI proteins. Both GPI proteins with an impaired GPI-SS as well as mutants defective in GPI anchor attachment could be employed for such an analysis.

## 1.4. GPI anchor attachment onto GPI precursor proteins

### 1.4.1. Introduction

As described in the previous section, proteins destined to receive a GPI anchor are synthesized as precursor proteins with an N-terminal signal sequence that targets the protein to the endoplasmic reticulum and a C-terminal GPI signal sequence (GPI-SS) which is recognized by the GPI:protein transamidase (Amthauer *et al.*, 1993). GPI anchor attachment occurs during or soon after translocation of a GPI precursor protein, and results in substitution of the C terminal GPI-SS downstream of the  $\omega$  site by a preformed GPI anchor glycolipid (Udenfriend and Kodukula, 1995). Although both substrates for GPI anchor attachment, the GPI anchor and several GPI precursor proteins, as well as the transamidation reaction had been described some time ago, the GPI:protein transamidase remained undiscovered (Udenfriend and Kodukula, 1995). 5 years ago, two putative components of the transamidase could be isolated by complementing two yeast mutants both accumulating GPI precursor glycolipids and an immature form of the GPI model protein Gas1 (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996). Recently, these two proteins – when overexpressed - were shown to interact with each other implying that they are components of the GPI:protein transamidase (Ohishi *et al.*, 2000; Fraering *et al.*, submitted).

### 1.4.2. The GPI signal sequence

The GPI signal sequence (GPI-SS) of several GPI precursor proteins was intensively explored biochemically by mutational analysis and computationally, i.e. by comparing natural GPI-SSs (Gerber *et al.*, 1992; Kodukula *et al.*, 1993; Nuoffer *et al.*, 1993; Eisenhaber *et al.*, 1998). In terms of physico-chemical properties, the overall structure of the C terminal GPI-SS is conserved among all eucaryotic GPI proteins described so far. GPI-SSs are tripartite, i.e. they consist of the region around the cleavage/attachment site, also called the small amino acid domain (SAD; Aceto *et al.*, 1999), a short hydrophilic spacer region and a hydrophobic domain at the extreme C terminus (Moran and Caras, 1991a). Nevertheless, GPI-SSs are degenerate, i.e. they do not show a consensus sequence and are at least to some extent organism-specific (Moran and Caras, 1994; White *et al.*, 2000; Takos *et al.*, 2000). The GPI-SS of various GPI proteins from different eucaryotes are depicted in Figure 4. Based on their quite well-described properties, functional GPI-SSs were optimized and/or synthetically designed (Coyne *et al.*, 1993; Bucht *et al.*, 1999; Caras and Weddell, 1989). GPI-SS of all GPI proteins described so far are located at the very C terminus. However, it has been shown for a hDAF/hGH fusion protein that an internally positioned GPI-SS can direct GPI attachment as well (Caras, 1991a).

	$\omega$
yGas1	SSSASSSSSSSKKN <u>AATNVKANLAQVVFTSIISLSIAAGVGFALV</u>
yYap3	STASATSTSSKRN <u>VGDHIVPSLPLTLISLLFAFI</u>
yCwp1	IQAPNTVYEQTEN <u>AGAKAAVGMGAGALAVAAAYLL</u>
hDAF	HETTPNKGSGTTS <u>GTTRLLSGHTCFTLTGLLGLTLVTMGLLT</u>
hPLAP	TACDLAPPAGTTD <u>AAHPGRSVVPALLPLLAGTLLLLLETATAP</u>
hCD59	KKDL CNFNEQLEN <u>GGTSLSEKTVLLLVTPLAAAWSLHP</u>

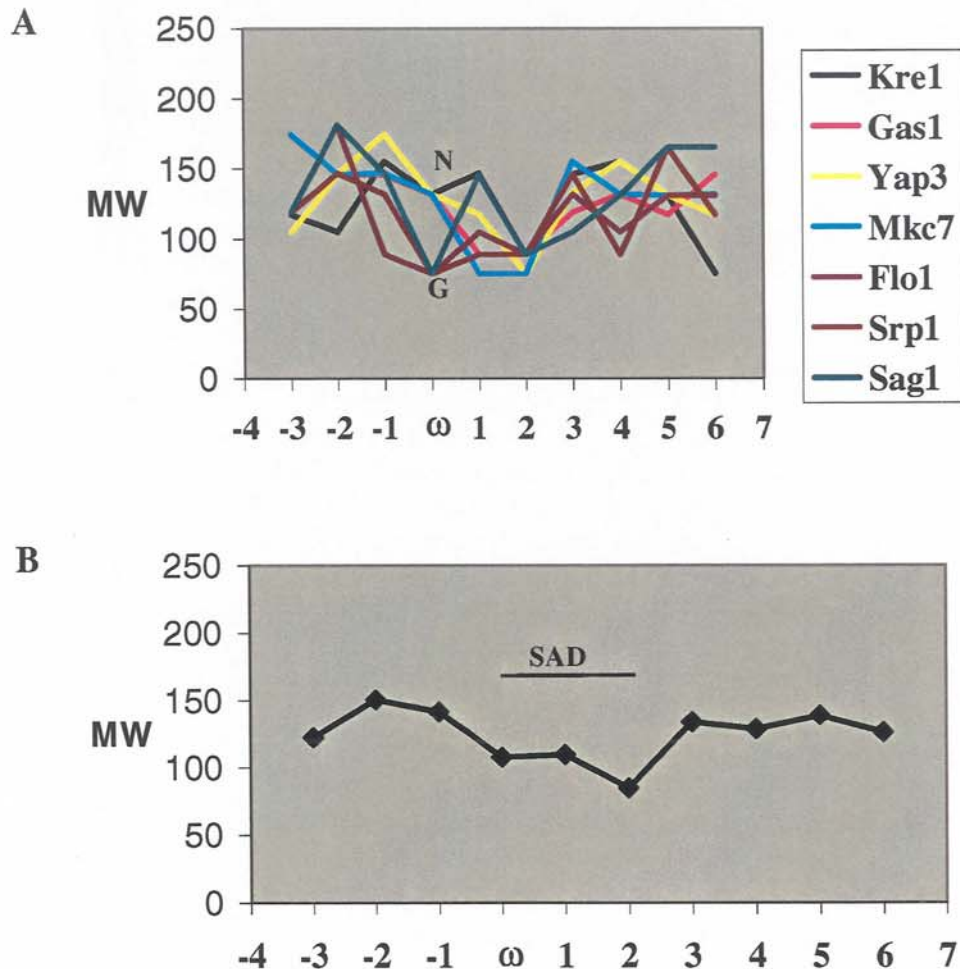
hFR $\alpha$	NEEVARFYAAAMS	<b>GAGPWA</b> <u>AWPFLLSLALMLLWLLS</u>
u-PAR	CNHPDLDVQYRSG	<b>AA</b> <u>PQPGPAHL</u> SLTITLLMTARLWGGTLLWT
r5'-NT	VIYPAVEGRIKFS	<b>TG</b> <u>SHCHGSFSLIFLSLWAVIFVLYQ</u>
rThy-1	KTINVIRDKLCKC	<b>GG</b> <u>ISLLVQNTSWLLLLLLLSLSFLQATDFISL</u>
Prion	QKESQAYYDGRRS	<b>SA</b> <u>VLFS</u> SPPVILLISFLIFLMVG
N-CAM	AFRTSAQPTVIPA	<b>TL</b> <u>GSPSTSSSFVSLLLSAVTLLLLC</u>
AChE	KLLNATACDGELS	<b>SS</b> <u>GTSSSKGIIFYVLFSILYLIFY</u>
PARP	EPEPEPEPEPEPG	<b>AAT</b> <u>LKSVALPFAIAAAALVAAF</u>
VSG	ESNCKWENNACKD	<b>SS</b> <u>ILVTKKFALTTTVSAAFVALLF</u>

**Figure 4. The GPI-SS of well described GPI proteins from various eucaryotic origin.**

The C terminal part of the GPI proteins is depicted with the small amino acid domain (SAD; in bold), the hydrophilic spacer region (waves) and the C terminal hydrophobic domain (underlined). The cleavagae/attachment site has been biochemically verified or predicted (Nuoffer *et al.*, 1991; Eisenhaber *et al.*, 1998; Udenfriend and Kodukula, 1995; Englund, 1993). yGas1: yeast glycolipid-anchored surface glycoprotein; yYap3: yeast aspartic protease-3 (Zhang *et al.*, 1997); yCwp1: yeast cell wall protein-1 (van der Vaart *et al.*, 1995); hDAF; human decay accelerating factor; hPLAP: human placental alkaline phsophatase; hCD59: human cluster of differentiation 59; hFR $\alpha$ : human folate receptor-alpha; u-PAR: human urokinase plasminogen activator surface receptor; r-5'-NT: rat 5'-nucleotidase; rThy-1: rat Thy-1 antigen; Prion: golden hamster prion protein; N-CAM: chicken neural cell adhesion molecule; AChE: *Torpedo californica* Acetylcholine esterase; PARP, VSG: *Trypanosoma brucei* variant surface glycoprotein and procyclic acid repeat protein, respectively; (Furukawa *et al.*, 1997 and references therein).

#### 1.4.2.1. The SAD domain

The small amino acid domain (SAD) is the region around the cleavage/attachment site onto which a complete GPI precursor glycolipid is added. As it can be inferred from its name, the SAD consists of an amino acid sequence of generally three, sometimes up to five small amino acids (Moran *et al.*, 1991a; Moran and Caras, 1991a; Coyne *et al.*, 1993). The GPI-SS cleavage/GPI anchor attachment site is generally found at the first position of the SAD and is called  $\omega$  (omega) site (Udenfriend and Kodukula, 1995). Figure 5 shows the SAD domain of 7 well-known yeast GPI proteins.



**Figure 5. The small amino acid domain of 7 well-known yeast GPI proteins.**

The molecular weights (MW) of the amino acids at positions around the  $\omega$  site are plotted. Amino acid residue positions are given with respect to the cleavage/attachment site ( $\omega$  site). The two points of convergence represent the most frequently observed  $\omega$  site amino acids found in yeast GPI proteins, asparagine (N) and glycine (G). The  $\omega+1$  site shows less stringent restriction for the size of the amino acid (Kre1 has even glutamic acid and  $\alpha$ -agglutinin (Sag1) lysine at this position). In contrast, the  $\omega+2$  position is more stringent and again consists of a small amino acid (see intersections at a low molecular weight level; panel A). Panel B shows the averaged MWs of all 7 GPI proteins depicted in panel A. Plotting of the van der Waals' volume against the same amino acid positions gives similar curves as presented for the molecular weight (not shown). Data were extracted from the yeast GPI protein database (cf. chapter 3.6.2.).

Extensive site-directed mutagenesis in different GPI-anchored proteins such as human placental alkaline phosphatase (Gerber *et al.*, 1992; Kodukula *et al.*, 1993), human decay accelerating factor (Moran *et al.*, 1991b) and yeast Gas1p (Nuooffer *et al.*, 1993) has elucidated that the potential  $\omega$  site is restricted to amino acid residues with

small side chains, namely Ala, Asn, Asp, Cys, Gly and Ser, whereas the  $\omega+1$  site can be roughly any residue except Pro; in contrast, the  $\omega+2$  site again requires an amino acid with a small side chain (Figure 4). Figure 6 pinpoints sequence amino acid preferences for the  $\omega$  site position as found by saturation mutagenesis for the above mentioned GPI proteins. As it can be seen, results obtained for the yGas1p are similar but not identical with the findings on the two human proteins.

**Table 2. Amino acid preferences for the  $\omega$  site.**

Protein	decreasing preference
yGas1p	N > S, G > A, D > C
hPLAP	N, S > D, G, A > C
hDAF <sup>Φ</sup>	S, A, D, N, G

Systematic mutation analysis data were summarized from the following sources: yeast Gas1p (Nuoffer *et al.*, 1993), human placental alkaline phosphatase (Micanovic *et al.*, 1990) and human decay accelerating factor (Moran *et al.*, 1991a). <sup>Φ</sup>For hDAF, quantitative differences between the efficiencies of the 5 amino acids could not be precisely extracted from the published data. The 5 amino acids giving relatively high levels of GPI-anchored DAF are listed.

The preference for a particular amino acid in the  $\omega$  site may depend on the species, the cell type and the particular protein being investigated because differences were obtained between different cells (e.g. yeast and human) and between two different human proteins (hPLAP and hDAF) analyzed in the very same cell type (Table 2). It is amazing that cells apparently do not always use for a given protein the amino acid which is the most efficient for GPI anchor addition; analysis of hPLAP showed that asparagine and serine are more preferred than the naturally occurring aspartic acid (Table 2 and 4); yGas1p, however, uses the optimal  $\omega$  site amino acid. Interestingly, it also has been shown that the natural GPI-SS of human acetylcholinesterase, as that of hPLAP, can be optimized for GPI modification by mutating the residues  $\omega+1$  and  $\omega+2$  (Bucht *et al.*, 1999). It is still mysterious why nature uses apparently "suboptimal" GPI signal sequences. Furthermore, it is also not known whether GPI protein precursors might be modified under physiological conditions at more than one site by a GPI anchor or not. At least, it was suggested by mutagenizing the GPI-SS of a miniPLAP/uPAR fusion protein that a GPI anchor might be added onto an amino acid residue of the SAD being different from the natural  $\omega$  site of uPAR (Aceto *et al.*, 1999).

The  $\omega+1$  site of hPLAP can tolerate fairly large amino acids with only a small reduction of GPI anchoring efficiency, whereas the amino acid preference at the  $\omega+2$  site was A, G > S > T > D > V; E, H, and P were totally inactive. Proline was the only amino acid that totally abolished GPI anchoring when present at either of the  $\omega$ ,  $\omega+1$ , or  $\omega+2$  site (Micanovic *et al.*, 1990; Kodukula *et al.*, 1993). For yGas1p, the analysis of the  $\omega+1$  and  $\omega+2$  amino acid positions were consistent with the results found on hPLAP (Nuoffer *et al.*, 1993).

Taken together, it has been shown by site-directed mutagenesis that all three amino acid positions,  $\omega$ ,  $\omega+1$ , and  $\omega+2$ , influence the efficiency of GPI anchoring and in addition, that the effects of mutagenesis are cumulative, in a negative and positive manner, since a less than optimal amino acid in one of these three positions could be

compensated – at least to some extent - by an optimal amino acid in another position (Nuoffer *et al.*, 1993). The latter finding might be a lead to understand the fact that suboptimal positions are found at these positions in natural processing sites (Gerber *et al.*, 1992). Recently, it has even found that a proline at the  $\omega+1$  site can be tolerated if glycine is present at position  $\omega+2$  (Bucht *et al.*, 1999). However, it still remains elusive why certain GPI proteins naturally do not employ the optimal GPI-SS as considered as a whole (Bucht *et al.*, 1999).

#### 1.4.2.2. The hydrophilic spacer region

In contrast to the SAD, far less is known about the specific requirements for the short hydrophilic spacer region. Its length has been estimated to approximately 6-10 or 8-12 amino acids residues depending on whether the positions  $\omega+1$  and  $\omega+2$  are included or not (Kodukula *et al.*, 1993; Coyne *et al.*, 1993; Furukawa *et al.*, 1994; Caro *et al.*, 1997); however, a few proteins such as hPLAP show spacer regions which are slightly shorter (Figure 4). Rather the overall physico-chemical properties of the spacer region and its length – at least to a certain extent - might be important for GPI anchor attachment than the single amino acid residues at defined distance from the  $\omega$  site or from the C terminus (Coyne *et al.*, 1993). Indeed, it has been shown for 5'-nucleotidase of bovine liver that the length of the spacer sequence may influence the efficiency of GPI modification. Systematic deletion and Ala insertion of the 8 amino acids-long spacer sequence of 5'-nucleotidase revealed that the length compatible for GPI anchor attachment ranges from 6 to 14 amino acids (Furukawa *et al.*, 1997). Furthermore, it has been reported for yeast Gas1p that deletants of the spacer region were less efficiently modified by a GPI anchor (Nuoffer *et al.*, 1993). However, as described in the latter study, removal of 2 amino acids gave a less pronounced effect on GPI anchoring than the deletion of only 1 amino acid residue. This is not consistent with a simple distance requirement hypothesis postulating that a certain minimal and maximal distance from the start of the hydrophobic domain must exist in order to correctly position the  $\omega$  residue with respect to the active site of the GPI:protein transamidase.

Concerning the physico-chemical properties of the spacer region, the precise requirements remain unclear although mutational studies were performed (Nuoffer *et al.*, 1993). Apparently, a broad range of different physico-chemical properties govern GPI modification, since it has been reported that several different nonfunctional sequences could be converted to a signal for GPI anchor attachment if a pair of small amino acids were positioned 10-12 residues N terminal to the hydrophobic domain (Moran and Caras, 1991b; Moran and Caras, 1991a). The lack of sequence conservation in the spacer region may reflect the large number of possible ways to fold into a structure compatible for recognition of the SAD domain by the transamidase; and these different ways of folding could explain why the sequence length between the SAD domain and the C terminal hydrophobic domain as well as the physico-chemical features are not tightly conserved. Alternatively and although being less probable, it might be speculated that the transamidase chooses another amino acid ( $\omega$ ) to add a GPI anchor as a consequence of varying the length and/or amino acid sequence of the spacer region. Interestingly, as it has been shown for SAD amino acid residues, a naturally occurring spacer region can be synthetically optimized for GPI modification by mutagenesis (Bucht *et al.*, 1999).

#### 1.4.2.3. The hydrophobic domain at the very C terminus

The C terminal hydrophobic domain is required to anchor a GPI protein precursor into the membrane of the endoplasmic reticulum, the site of GPI anchor attachment (Amthauer *et al.*, 1993). Its length ranges generally between 8 and 31 amino acids (Coyne *et al.*, 1993; Furukawa *et al.*, 1997). GPI-SS mutants lacking a C terminal hydrophobic domain are not modified by a GPI anchor (Caras *et al.*, 1989; Udenfriend and Kodukula, 1995). To be functional, the hydrophobic domain of the GPI-SS has been shown to require a moderate overall hydrophobicity and a minimum length (Caras, 1991b). Substitution with a single charged amino acid residue at any position in this region results in impairment of its functions (Lowe, 1992). Furthermore, as already discussed in the context of the hydrophilic spacer region, the hydrophobic domain must maintain a minimum distance from the  $\omega$  site (Coyne *et al.*, 1993). All these characteristics are reminiscent of the hydrophobic domain of the amino-terminal leader peptide for translocation of proteins into the ER. Indeed, it has been reported that replacement of the C terminal hydrophobic domain of hDAF with a signal peptide that normally functions in membrane translocation of a secretory protein, or with a random hydrophobic sequence, results in efficient and correct processing, producing GPI-anchored DAF at the cell surface (Caras and Weddell, 1989). However, the hydrophobic domain of GPI-SSs lack positively charged amino acids which are generally found after the hydrophobic sequence of typical transmembrane domains. Furthermore, it has been described in detail that the hydrophobic domains in the GPI-SS and the N terminal leader peptides have similar structural requirements (Yan *et al.*, 1998). Thus, as for the hydrophilic spacer region, overall physico-chemical properties and the length appear to be more important for the proper function of the hydrophobic domain than the precise amino acid sequence.

#### 1.4.2.4. The $\omega$ -minus region

In contrast to the sequence located C terminally to the cleavage/attachment site, the region upstream of the  $\omega$  site, the so-called  $\omega$ -minus region, is not a prerequisite for GPI anchor attachment (Coyne *et al.*, 1993).

However, this region has been reported to participate in subcellular localization of yeast GPI proteins. Two kinds of amino acid sequence signals have been proposed as being responsible for the selection: dibasic residues for remaining on the plasma membrane as determined by sequence analysis (Caro *et al.*, 1997; Vossen *et al.*, 1997), and specific amino acid residues at the sites  $\omega$ -4 or  $\omega$ -5 together with the site at the position  $\omega$ -2 for incorporation into the cell wall as determined by mutational analysis (Hamada *et al.*, 1998a). These two hypotheses were analyzed by using both synthetic model sequences and GPI-SS mutants of authentic yeast GPI proteins. Amino acid residues V, I, or L at the  $\omega$ -4/5 site and Y or N at the  $\omega$ -2 site were found to stimulate cell wall incorporation, whereas the basic residues R and/or K in the short  $\omega$ -minus region diminished cell wall incorporation, such that the proteins remained on the plasma membrane as GPI-anchored proteins (Hamada *et al.*, 1999).

However, the dibasic model has been recently questioned by a study (De Sampaio *et al.*, 1999) which reported that  $\alpha$ -galactosidase constructs having at their C terminus the GPI-SS together with the  $\omega$ -minus region of the yeast proteins Gas1 and Yap3



which both contain the putative dibasic motif at the positions  $\omega$ -1 and  $\omega$ -2 and were formerly shown to be located to the plasma membrane (Nuoffer *et al.*, 1991; Ash *et al.*, 1995) were covalently incorporated into the cell wall to a considerable extent. These two contradictory studies (Hamada *et al.*, 1999; De Sampaio *et al.*, 1999) might indicate that either the proteins were mislocalized based on the modified structure of synthetic fusion proteins and/or that additional signals could be involved in subcellular localization of GPI proteins. Candidates for additional signals might be other amino acid positions in the  $\omega$ -minus region, different N- and O-glycosylation patterns of mature GPI proteins, or the presence of different GPI anchor structures. In addition, it has to be mentioned, certain analyses on yeast cell wall proteins can be quite demanding because of the complex posttranslational modifications such as glycosylation which might lead to blotting artefacts as reported (van der Vaart *et al.*, 1996). Further studies are required to precisely define the requirements which govern subcellular localization of yeast GPI proteins. At least it has been demonstrated that the  $\omega$ -minus region participates in this process (Hamada *et al.*, 1999).

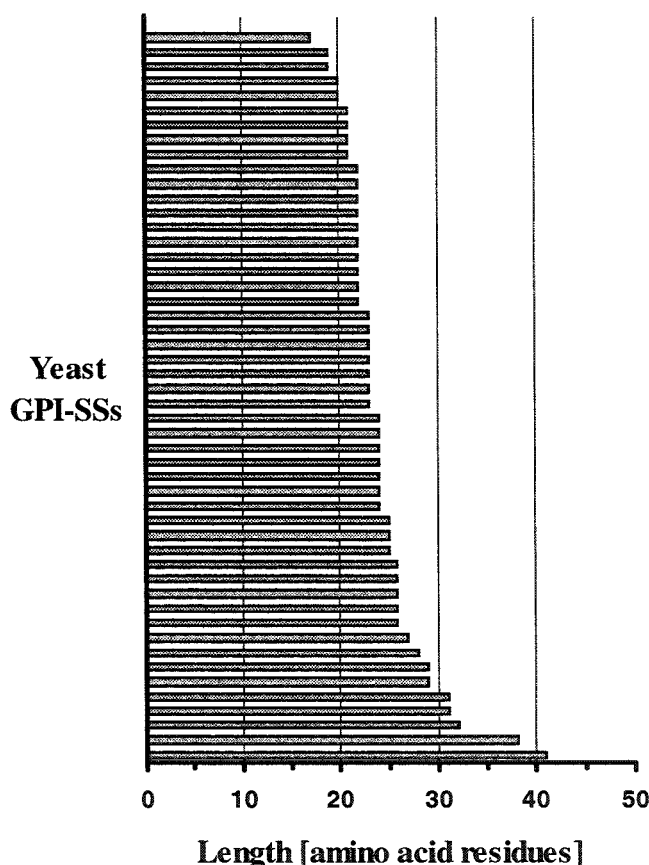
#### 1.4.2.5. Computational analysis of the GPI-SS sequence

##### *Analysis of eucaryotic GPI-SSs*

Sequence properties of the GPI-SS were also defined by computational comparison of GPI protein sequences accumulated in databases (Eisenhaber *et al.*, 1998). The dataset comprised 155 sequences of various origin: 104 metazoan, 40 mostly parasitic protozoan, 10 fungi and 1 herpes virus protein. The total GPI-SS sequence length of most of the proteins clustered between 18 and 29 amino acid residues, whereby the protozoan GPI-SS was slightly shorter (4 to 8 residues) than that of metazoan proteins as already reported previously (Caras and Moran, 1994). The GPI modifying motif was structured into four regions (Figure 4): 1. an unstructured linker region ranging from  $\omega$ -11 to  $\omega$ -1; 2. a region of small residues  $\omega$ -1 to  $\omega$ +2, of which the most frequent amino acid residue for the  $\omega$  site in both protozoa and metazoa was S; 3. a spacer region ( $\omega$ +3 to  $\omega$ +8) of moderately polar residues with a possible hydrophobic island at position  $\omega$ +4 and  $\omega$ +5; and 4. a leucine-rich hydrophobic tail beginning with  $\omega$ +9 up to the C terminal end. Furthermore, 60 % of metazoan (but not protozoan) proteins showed at position  $\omega$ +15 either S, T or A. Concerning the regions 2, 3 and 4, the results are generally consistent with previous observations and experimental data. However, the region from  $\omega$ -11 to  $\omega$ -1 - although poorly explored - was never reported to be required for GPI anchor attachment (Coyne *et al.*, 1993). Eisenhaber *et al.* (1998) proposed a model in which this region represents a linker sequence connecting the folded substrate protein with the  $\omega$  site being hidden by catalytic residues deep inside the active enzyme. It would be of interest to test this hypothesis experimentally, e.g. by replacing this  $\omega$ -minus region with a peptide with strong internal  $\alpha$ -helical preference. Furthermore, the authors propose a model for the GPI-SS binding pocket of the putative transamidase based on their findings (cf. section 1.4.4.2, Figure 9).

### ***Analysis of the yeast GPI-SS***

About 70 GPI proteins were predicted for the yeast proteome (Caro *et al.*, 1997; Hamada *et al.*, 1998; YPD, 2000; cf. chapter 3.6.2.); 50 of them were experimentally shown to be GPI-anchored. Analysis of these 50 GPI proteins gave a mean length for the complete GPI signal sequence of 24.4 amino acids residues. The GPI-SS length of all the proteins is depicted in Figure 6. Extremes were the GPI-SSs of YDR134c, Yps7 and Exg2 displaying a length of 17, 38 and 41 amino acid residues. The length of about 85% of the analyzed GPI-SSs was in a range of 20 to 30 amino acid residues. However, one has to consider that these data were calculated mainly based on predicted  $\omega$  site positions and therefore should be considered as a tentative overview on yeast GPI proteins.



**Figure 6. GPI-SS length of 50 experimentally verified yeast GPI proteins.**

The length of GPI signal sequences were extracted from the yeast GPI protein database (Chapter 3.6.2.). Depicted are only GPI-SSs of experimentally verified yeast GPI proteins. For most of the sequences, the length has been determined by predicting the  $\omega$  site position.

As predicted by Caro *et al.* (1997), most of the yeast GPI proteins are suggested to have N or G at their  $\omega$  site; analysis by using the big-PI-Predictor proposes that a few yeast GPI proteins might also use S, A, or D as cleavage/attachment site (cf. chapter 3.6.2.). The hydrophilic spacer region of most of the yeast GPI proteins generally comprises 6 to 10 amino acids (counted by excluding the positions  $\omega+1$  and  $\omega+2$ ), and the length of the hydrophobic region amounts to approximately 11 to 20 residues. Therefore it can be concluded that GPI-SSs of yeast proteins have similar sequence requirements as reported for several other eucaryotic GPI proteins.

#### 1.4.2.6. Prediction of GPI-SSs and the $\omega$ site

Nowadays, more than 350 eucaryotic GPI proteins were described or predicted, and for more than half of them experimental data prove the presence of a GPI anchor (Kodukula *et al.*, 1995; Caro *et al.*, 1997; Hamada *et al.*, 1998b; Furukawa *et al.*, 1997; Eisenhaber *et al.*, 2000; Eisenhaber *et al.*, 1998). Experimental verification of a protein being GPI-modified at a given site requires an expensive laboratory effort. The most direct approach includes sequencing of the protein itself. This involves its proteinase digestion into smaller peptides, the separation of the peptides with a GPI group and the physico-chemical characterization of this peptide and the GPI-modified amino acid residue with methods such as radioactive labeling, chemical peptide sequencing, composition analysis, NMR, mass spectrometry (Ferguson *et al.*, 1988; Nuoffer *et al.*, 1991; Sugita and Masuho, 1995; Kodukula *et al.*, 1995). This approach has been performed for only a few GPI proteins including yeast Gas1p, human and rat 5'-nucleotidase, hDAF, human CD59, *Trypanosoma brucei* VSG and PARP, human placental alkaline phosphatase, renal dipeptidase and hamster prion protein (Eisenhaber *et al.*, 1998, and references therein). Rat Thy-1 was the first mammalian GPI protein for which the  $\omega$  site and the complete GPI structure was determined (Homans *et al.*, 1998; Conzelmann *et al.*, 1988a). Easier is the experimental verification for the presence of a GPI anchor. Proteins are generally analyzed for the presence of a radiolabeled GPI-specific component such as *myo*-inositol and dihydrosphingosine (Sipos *et al.*, 1994b; Reggiori *et al.*, 1997) or for the presence of a cyclic inositol-phosphate structure detected with anti-CRD antibodies after treatment with PI-PLC (Zamze *et al.*, 1988).

In terms of genome-wide analysis, prediction of GPI-anchored proteins is a useful tool and based mainly on the presence of both an N terminal signal sequence and the presence of a C terminal GPI-SS. Algorithms for the prediction of a GPI signal sequence alone or together with its putative  $\omega$  site have been established based on experimental data and computational sequence comparison as described in the previous sections (Eisenhaber *et al.*, 1999; Caro *et al.*, 1997; Udenfriend and Kodukula, 1995). Software to predict the presence of a GPI-SS in an unknown sequence (PSORT) and in addition to predict the most probable cleavage/attachment site (big-PI Predictor) can be accessed by the internet (for www sites see chapter 7).

#### 1.4.2.7. Specificity of GPI-SSs in eucaryotic kingdoms

Despite the common evolutionary features of GPI-SSs, there appears to be sufficient heterogeneity to affect the efficiency of processing of signals in different eucaryotic kingdoms. The existence of these variations could be exploited in the design of small inhibitor molecules that distinguish between the mammalian and protozoan GPI transamidase. Protozoan GPI-SSs for example are poorly processed in mammalian cells (Moran and Caras, 1994) and slime moulds (Reymond *et al.*, 1995). The *T. cruzi* surface glycoprotein gp82 became not GPI-anchored in COS-1 cells (Ramirez *et al.*, 1999), and MSP-1 of *Plasmodium falciparum* did not undergo GPI anchor addition in mammalian cells (Yang *et al.*, 1999; Burghaus *et al.*, 1999). In contrast, human melanotransferrin p97 was recognized by the GPI anchoring machinery of certain insects (Hegedus *et al.*, 1999). The plant *Nicotiana tabacum* has been described to

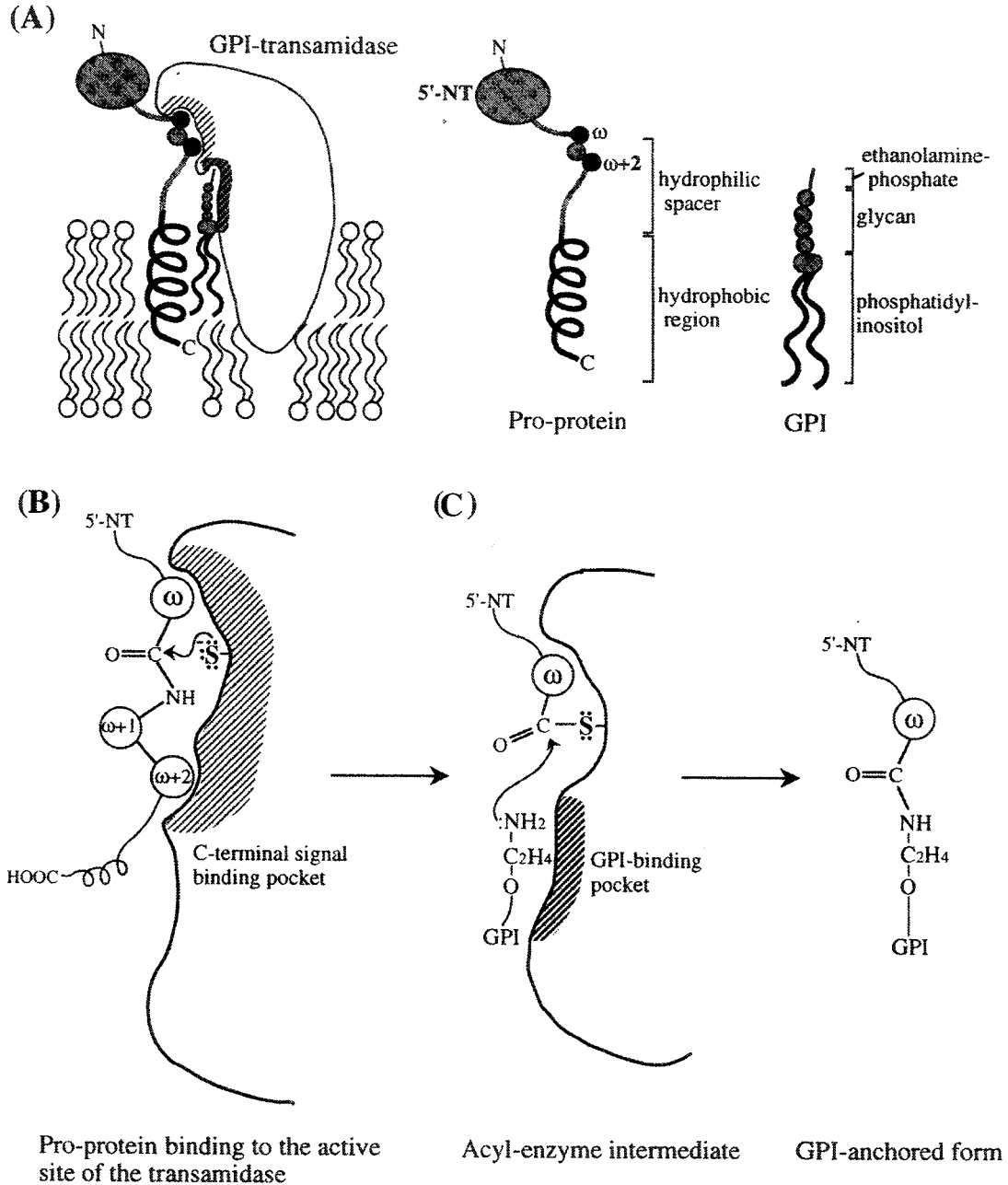
recognize the GPI-SS of the yeast GPI protein Gas1, but not that of mammalian Thy-1. Yeast and mammalian signals appear to be more closely related, whereby the GPI anchoring machinery of yeast appears to have a more restricted specificity (Guadiz *et al.*, 1998; Morel and Massoulie, 1997). The GPI-SS of human placental alkaline phosphatase was not recognized by the yeast GPI anchoring machinery (De Sampaio *et al.*, 1999; Meyer *et al.*, in preparation), whereas rat acetylcholinesterase was well expressed and processed in the yeast *Pichia pastoris* (Morel and Massoulie, 1997).

### 1.4.3. The Transamidation reaction

After completion of its biosynthesis, a complete GPI precursor glycolipid is attached to the  $\alpha$  carbonyl group of the  $\omega$  amino acid residue by forming an amide bond with the amino group of the ethanolaminephosphate moiety present on the third mannose of the GPI anchor, a transfer resulting in removal of the C terminal GPI-SS (Udenfriend and Kodukula, 1995). It is not known whether also ethanolaminephosphate groups on other mannoses of the GPI anchor core structure might be modified by such an amide linkage. However, the fact that the deletant of *YLL031c* and the mutant *gpi10-1* do not anchor via the EtN-P groups on the first and second mannose makes this hypothesis unlikely. GPI anchor addition occurs during or soon after translocation across the membrane of the endoplasmic reticulum (Mayor *et al.*, 1991). Recognition of the C terminal GPI-SS requires translocation of its hydrophobic domain across the ER membrane (Wang *et al.*, 1999). Furthermore, upon entry into the ER, GPI proteins interact prior to the GPI transfer reaction with the molecular chaperone BiP in an ATP-dependent manner as described for mammalian cells (Amthauer *et al.*, 1993). The transfer reaction is thought to occur by a transamidation reaction which implies the formation of an activated carbonyl intermediate. GPI anchor addition is then completed by a nucleophilic attack of the amino group of the ethanolamine moiety of the GPI anchor on the activated carbonyl group (Furukawa *et al.*, 1997; for a precise description see Figure 7). Although the formation of a carbonyl-enzyme intermediate has been strongly suggested by experiments in a cell-free system (Maxwell *et al.*, 1995a), the precise sequence of events is not yet elucidated. For example, it is not known whether both substrates, the GPI precursor protein and the GPI anchor are bound to the transamidase at the same time. However, the formation of the carbonyl intermediate has been reported not to be dependent on the presence of a GPI lipid (Maxwell *et al.*, 1995a).

Several findings support the hypothesis that GPI anchor attachment acts as a transamidation reaction: first, small amounts of cleaved but not GPI-anchored proteins are detected in microsomal systems (Amthauer *et al.*, 1992) and the GPI-SS cleavage is strongly stimulated by the presence of small nucleophilic agents (Maxwell *et al.*, 1995a), both observations indicate the formation of a carbonyl intermediate; second, in microsomal preparations of *Trypanosoma brucei*, GPI modification of unprocessed, but appropriately translocated VSG polypeptides was restored by the addition of *in vitro* synthesized or exogenously supplied GPI anchors; ATP or GTP were not required for GPI anchor attachment in this system. ATP or GTP would be a prerequisite for a multi-step mechanism comprising proteolytic cleavage of the GPI-SS, activation of the processed peptide or the GPI anchor and transfer of the GPI anchor (Mayor *et al.*, 1991). Third, in living cells, one cannot observe biosynthetic

intermediates from which the GPI-SS has been removed, but to which a GPI has not been added. This holds also true for mammalian mutant cell lines unable to synthesize complete GPIs (Conzelmann *et al.*, 1988a; Conzelmann *et al.*, 1986).

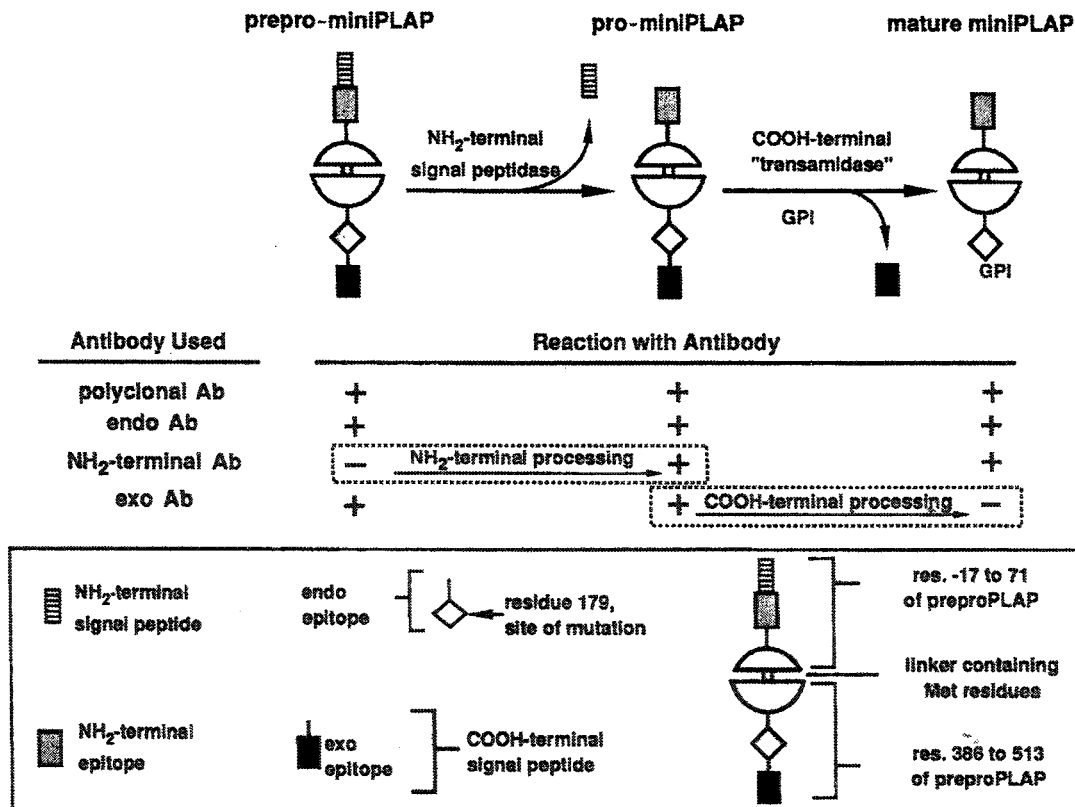


**Figure 7. Hypothetical mechanism of the transamidation reaction.**

The transamidation reaction is depicted for the mammalian GPI protein 5'-nucleotidase and might occur similarly for all other GPI proteins in other eucaryotes. **A: Recognition and binding of the substrates:** The GPI precursor protein and the complete GPI precursor glycolipid being both substrates of the GPI:protein transamidase may bind both to form an enzyme-substrates complex (E-S<sub>2</sub> complex) on the luminal side of the endoplasmic reticulum. Both the SAD domain of the GPI-SS on the GPI precursor protein and the ethanolaminephosphate moiety on the GPI anchor are presented to the active site of the

GPI:protein transamidase. **B: Formation of a carbonyl intermediate:** After formation of the E-S<sub>2</sub> complex, a carbonyl (acyl-enzyme) intermediate is formed between the active site residue cysteine of the catalytic subunit of the transamidase (Meyer *et al.*, 2000; Ohishi *et al.*, 2000) and the  $\alpha$  carbonyl group of the  $\omega$  site residue; the sulfur atom of the active site residue attacks temporarily the C atom of the carbonyl group and destabilizes the peptide bond between residues  $\omega$  and  $\omega+1$  resulting in the release of the C terminal sequence downstream of the  $\omega$  site. It is assumed that the sulfur atom is, at least transiently, negatively charged during the catalytic process. **C: Nucleophilic attack on the carbonyl intermediate and GPI anchor transfer:** The nitrogen atom of the amino group present on the ethanolamine phosphate moiety of the GPI anchor replaces the active site sulfur atom as ligand on the carbonyl C atom of the GPI-SS by a nucleophilic attack. This nucleophilic attack completes GPI anchor addition which results then in the release of the GPI-anchored protein from the transamidase. Modified scheme from Furukawa *et al.* (1997).

Cell-free reconstitution systems were developed to study the transamidation reaction with membranes of different organisms (Kodukula *et al.*, 1992; Doering and Schekman, 1997; Sharma *et al.*, 1999; Mayor *et al.*, 1991; Udenfriend and Kodukula, 1995; Kodukula *et al.*, 1995). Several model GPI proteins or GPI-SSs of them were used for these *in vitro* systems including DAF, VSG, Gas1 and PLAP. For the latter, a recombinant form reduced in size is widely used in an *in vitro* translation-translocation-GPI addition system: prepro-mini-PLAP (Figure 8). Furthermore, it has been reported that for cell-free assays, the GPI anchor can be replaced by hydrazine and hydroxylamine which are known nucleophilic acceptors in transamidase- and transpeptidase-catalyzed reactions (Maxwell *et al.*, 1995b). Carboxy-terminal processing of nascent polypeptides by the transamidase in the presence of hydrazine is governed by the same parameters as GPI addition (Ramalingam *et al.*, 1996).



**Figure 8. Scheme of cell-free processing of prepro-miniPLAP.**

The size of PLAP was reduced in order to detect more easily different processing intermediates. Radiolabeled and co-translationally processed prepro-miniPLAP can be immunoprecipitated by different epitope-specific antibodies and detected after SDS-PAGE separation by fluorography. Prepro-miniPLAP incubated with microsomal membranes is first processed by the N terminal signal peptidase which gives rise for pro-miniPLAP having a reduced size compared to prepro-mini-PLAP. Pro-miniPLAP - which lacks the N terminal signal sequence - is then processed by the GPI:protein transamidase resulting in the formation of GPI-anchored miniPLAP displaying a molecular weight in between that of pro-miniPLAP and free miniPLAP lacking both the N terminal signal peptide and the C terminal GPI-SS. miniPLAP modified by hydrazide instead of a GPI anchor migrates more or less the same way as free miniPLAP (Maxwell *et al.*, 1995a). (Kodukula *et al.*, 1995).

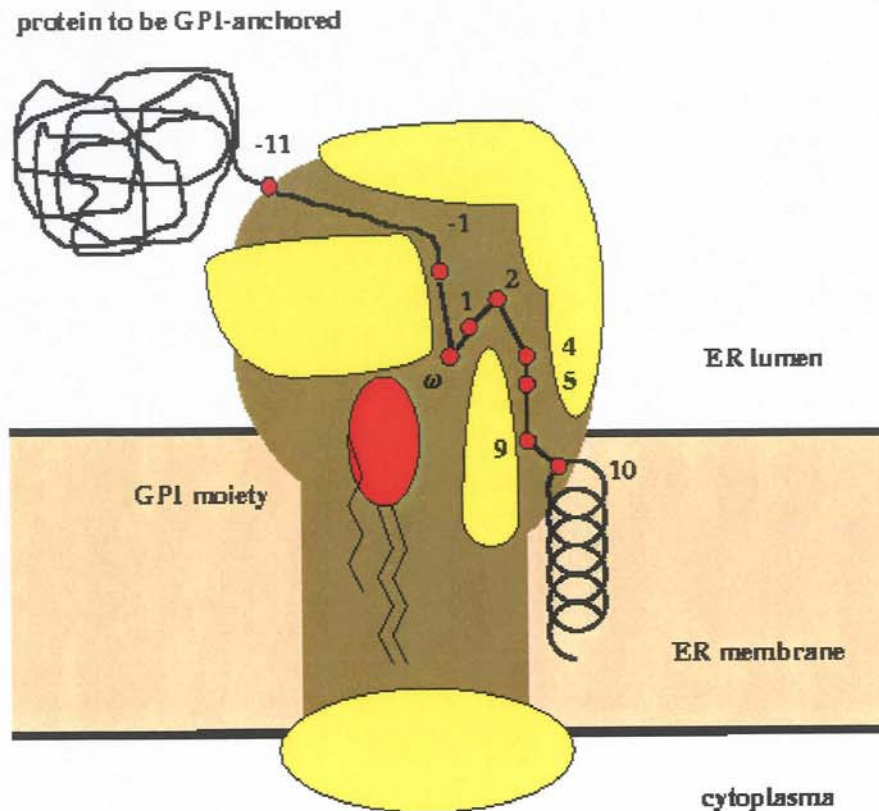
**1.4.4. The GPI:protein transamidase****1.4.4.1. Introduction and subcellular localization**

GPI anchor attachment occurs posttranslationally and in the lumen of the endoplasmic reticulum (Amthauer *et al.*, 1993). Thus, it was suggested that the putative GPI:protein transamidase is located on the luminal side of the ER membrane (Udenfriend and Kodukula, 1995). Experiments in both living cells and *in vitro* systems had revealed the catalytic activity of the putative transamidase (Udenfriend and Kodukula, 1995). The transamidase was proposed to be the key enzyme in GPI anchor attachment being responsible for the recognition of the substrates (the preformed GPI anchor and a GPI precursor protein) and for the catalysis of the GPI transfer onto a GPI precursor protein resulting in removal of the C terminal GPI-SS (Udenfriend and Kodukula, 1995). During several years of GPI research, the GPI:protein transamidase remained undiscovered. Two putative subunits of the GPI:protein transamidase, Gaa1p and Gpi8p, were identified by complementing two yeast mutants accumulating complete GPI precursor glycolipids and the 105 kDa immature form of the yeast GPI model protein Gas1p (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996).

Since the GPI-SS differs between species, the protein recognizing this signal, the putative GPI:protein transamidase, is a potential target for chemotherapy (Tiede *et al.*, 1999).

**1.4.4.2. The binding pocket for the GPI signal sequence*****Computational analysis***

Since the transamidase complex is still insufficiently described, the GPI-SS binding pocket of the putative GPI:protein complex remains unknown. Nevertheless, precise analysis of GPI-SSs might already predict some properties before crystal structures of the GPI:protein transamidase or its components are available. The GPI-SS of 155 mainly metazoan GPI proteins were analyzed by computational comparison (Eisenhaber *et al.*, 1998; Figure 9; cf. section 1.4.2.5.).



**Figure 9. Structural scheme for the putative GPI:protein transamidase.**

The putative transamidase is thought to be a protein with a large membrane and an ER-luminal domain. The hypothetical structure of the putative GPI:protein complex was predicted by analyzing the structure of GPI-SSs (Eisenhaber *et al.*, 1998). The preformed GPI anchor (red oval) as well as the hydrophobic domain of the GPI-SS (helix) are inserted into the membrane of the endoplasmic reticulum. Amino acid residues predicted to be important for complex formation with the transamidase (red circles) are depicted with numbers defining the distance from the  $\omega$  site. The catalytic cavity comprises residues  $\omega$ -1 to  $\omega$ +2 and should communicate with the GPI-moiety binding site. The channel between the substrate protein and the catalytic site is occupied by a flexible polypeptide segment from  $\omega$ -11 to  $\omega$ -1. The spacer ( $\omega$ +3 to  $\omega$ +9) with a possible specific binding site for  $\omega$ +4 and  $\omega$ +5 links the catalytic cleft with the hydrophobic tail. Modified scheme from Eisenhaber *et al.* (1998).



taken as the major parameter dictating the amino acids in the region from  $\omega-1$  to  $\omega+2$ , this may explain the finding of Kodukula *et al.* (1993), who reported a high permissiveness of position  $\omega+1$  with respect to the amino acid type except for proline in the case of human placental alkaline phosphatase. The positions  $\omega-1$ ,  $\omega$  and  $\omega+2$  (T, D, A; Figure 4) have a total volume of about 327 Å<sup>3</sup>. Most residues are smaller than 200 Å<sup>3</sup> and could fit well into the remaining space of the catalytic cavity. Even tryptophan having a mean volume of approximately 232 Å<sup>3</sup> can enter, although with difficulty as the rate of GPI-modification of this mutant was 10 times lower than that of the wt (A at the  $\omega+1$  position; Kodukula *et al.*, 1993).

Nonetheless, restrictions for the catalytic cleft of the transamidase are not only defined by the volume since only about 6 amino acid types are allowed at the  $\omega$  site and since proline is not allowed at any of the positions  $\omega$ ,  $\omega+1$ , and  $\omega+2$ . Binding of this structure by the transamidase might be inhibited by steric hinderance; additionally, the  $\alpha$  carbonyl group of proline at the  $\omega$  site is considered as a bad nucleophilic acceptor (Maxwell *et al.*, 1995b; Ramalingam *et al.*, 1996). However, it has been proposed that introduction of a proline into certain SAD positions might shift the cleavage/attachment site (Aceto *et al.*, 1999). In addition, organism- and/or protein-specific interactions between SAD residues of the GPI protein precursor and transamidase amino acid residues reaching into the active site cleft might improve GPI anchor addition (Kodukula *et al.*, 1993; Nuoffer *et al.*, 1993).

Crystal structures of transamidase-substrate complexes and additional biochemical analysis of GPI anchor attachment reactions will provide more insight into the interactions of the transamidase with its substrates.

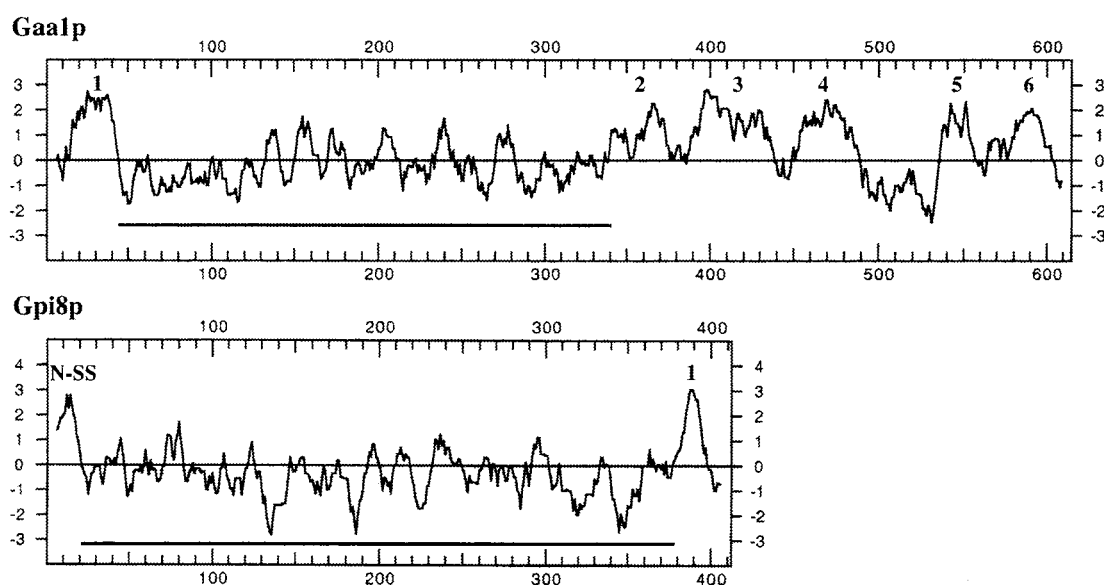
#### 1.4.4.3. Possible components of the putative yeast GPI:protein transamidase complex

Transamidase mutants were expected to accumulate both complete GPI lipids as well as GPI precursor proteins, a phenotype exhibited by two yeast mutants, *gaal* and *gpi8*, and a mammalian mutant cell line, class K (Mohney *et al.*, 1994b; Hamburger *et al.*, 1995; Benghezal *et al.*, 1995; Chen *et al.*, 1996). Complementing of the yeast mutants led to the identification of two putative components of the GPI:protein transamidase: Gaa1p and Gpi8p; the mammalian mutant was shown to be deficient in the function of human homolog of yeast Gpi8p, hGpi8p (Yu *et al.*, 1997). Recently, it has been shown that human Gaa1p and human Gpi8p both overexpressed in CHO cells form a protein complex suggested to be the or a part of the putative GPI:protein transamidase complex (Ohishi *et al.*, 2000). Although strong evidence for an interaction between Gaa1p and Gpi8p exists, it is not known whether this interaction is stable throughout the whole catalytic reaction or transient, i.e. occurs only at a certain stage of the catalytic reaction. Concluding evidence for the existence of a GPI:protein transamidase complex containing Gaa1p and Gpi8p as subunits might be provided by demonstrating a direct interaction of these putative subunits with a GPI protein precursor or a preformed GPI lipid.

In the following, Gaa1p and Gpi8p as possible subunits of the GPI:protein transamidase are put in front. Table 3 compares both proteins with each other.

#### 1.4.4.4. Gaa1p

Gaa1p is the acronym for GPI anchor attachment protein 1 and was discovered by complementing the temperature-sensitive yeast mutant *end2* with a *S. cerevisiae* genomic library. *end2* was isolated in a screen for endocytosis-deficient mutants being defective for accumulation of the endocytotic marker Lucifer yellow in the vacuole (Chvatchko *et al.*, 1986). However, since this mutant was deficient neither in internalization nor in degradation of the pheromone  $\alpha$ -factor, the negative effect on the endocytotic pathway was suggested to be indirect. Indeed, the primary effect of the mutation in *end2* was described to consist in an impairment of GPI anchor attachment since this mutant accumulated GPI precursor glycolipids and displayed retarded maturation of the GPI protein Gas1. Therefore, its name was changed from *end2* to *gaal* (Hamburger *et al.*, 1995). The *GAA1* gene is essential for yeast cell viability and encodes a 68 kDa ER protein with a large luminal domain, several transmembrane spanning domains, and an ER retrieval signal on its extreme C-terminus (Table 3).



**Figure 10. Kyte&Doolittle hydropathy plots of the yeast proteins Gaa1p and Gpi8p.**

The hydropathy plot of Gaa1p predicts the presence of 6 putative transmembrane domains (TMDs; enumerated from 1 to 6). Between the first and second TMD a large hydrophilic, ER-luminal domain of approximately 306 amino acid residues is predicted (bar). Gpi8p shows two transmembrane domains: one at the N terminus being the N terminal signal peptide (N-SS) which is suggested to be cleaved off by the signal peptidase upon entry of the growing peptide in the lumen of the ER and another C terminal domain (1) supposed to play a role in localization of the protein into the to the ER membrane. Hydrophilic domains are shown by bars. Kyte&Doolittle hydropathy plots were computed by using the DNA Strider™ 1.3f3 software.

It is very difficult to predict the exact role of Gaa1p in the process of GPI anchor attachment since no significant homologies to other proteins of known function were found. Its character as integral ER membrane protein with several membrane spanning domains led to the conclusion that Gaa1p might be involved in the recognition of the GPI-SS of newly made GPI precursor proteins. This possibility which is supported by the fact that overexpression of yeast Gaa1p could partially suppress the processing defect of GPI-SS mutants of Gas1p (Hamburger *et al.*, 1995).

Furthermore, Gaa1p could be involved in binding the complete GPI precursor glycolipid prior to be transferred onto a GPI protein precursor, in membrane anchoring of Gpi8p since Gpi8p lacking the C terminal transmembrane domain are fully functional and could be hold close to the ER membrane by interacting with Gaa1p as shown (Ohishi *et al.*, 2000), or eventually in several interactions with other ER membrane or ER luminal components such as for example with the Sec61 translocation pore and chaperones. However, no interactions of Gaa1p with other components than Gpi8p were shown so far. In addition, Gaa1p together with Gpi8p has been reported to be required for formation of the carbonyl intermediate (Ohishi *et al.*, 2000). Experimentally verified orthologs of yeast Gaa1p were reported in humans and mice (Table 3), whereas predicted orthologs were found by BLAST searches by which no putative plant ortholog could be found up to now. Orthologs showed a sequence identity of around 24 to 30 % over more than 350 (*C.elegans*), 500 (*H. sapiens*, *M. musculus*, *S. pombe*) and 600 (*D. melanogaster*) amino acid residues with the yeast Gaa1p protein.

**Table 3. Gaa1p and Gpi8p, two putative subunits of the yeast GPI:protein transamidase.**

Property	Gaa1p	Gpi8p
length	614	411
pI	6.02	5.08
codon bias	0.057	0.063
N terminal signal peptide	no N terminal signal peptide predicted, but there is an N terminal hydrophobic domain which might remain uncleaved	cleavable (between G <sup>21</sup> and A <sup>22</sup> )
Other motif for subcellular localization	KKKXX motif for ER retrieval	-
predicted transmembrane domains	6	1
subcellular localization (experimental data)	integral membrane protein of the ER; a large hydrophilic domain is in the ER lumen (N and C terminus are on the cytosolic side of the ER)	integral membrane protein of the ER; the large hydrophilic domain is in the ER lumen (N terminus in the ER lumen, C terminus is cytosolic)
N glycosylation	1 site (N <sup>87</sup> )	3 sites (N <sup>23</sup> , N <sup>256</sup> , N <sup>344</sup> )
predicted molecular weight (with N terminal signal sequence, without glycans)	69'221 kDa	47'402 kDa
molecular weight as	one band of 70 kDa	bands of 50, 48, 46

determined by SDS-PAGE of whole cell extracts deglycosylated with EndoH	68 kDa	(depending on extent of N glycosylation); a soluble band of 44 kDa 44 kDa
functional family	-	C13 family of cysteine proteases (hemoglobinase family); similarities to the active site of caspases (C14 family of cyteine proteases)
predicted function in GPI anchor attachment	recognition of the GPI anchor and/or the GPI-SS; anchoring of Gpi8p to the ER membrane; interaction with ER membrane components	putative catalytic subunit of the GPI:protein transamidase
Interaction	Gpi8p	Gaa1p
phenotype of the deletant	lethal	lethal
phenotype of the temperature-sensitive mutant at the non-permissive temperature	accumulation of GPI precursor glycolipids and of GPI precursor proteins; affects endocytosis	accumulation of GPI precursor glycolipids and of GPI precursor proteins; hypersensitive to CFW
complementation of deletants or mutants in other organisms	no data available	y <i>GPI8</i> complements the human class K defect if the region adjacent N terminally to the TMD (position 298 to 384) were replaced by the corresponding sequence (positions 305 to 371) of <i>hGPI8</i> (Ohishi <i>et al.</i> , 2000); yeast/human chimeric proteins cannot complement a yeast <i>gpi8</i> deletant strain (chapter 3.3.)
Yeast ORF	YLR088w	YDR331w
functional homologs: experimentally shown	<i>H. sapiens</i> , <i>M. musculus</i>	<i>H. sapiens</i> , <i>L. mexicana</i>
predicted <sup>#</sup>	<i>S. pombe</i> , <i>D. melanogaster</i> , <i>C. elegans</i>	<i>S. pombe</i> , <i>D. melanogaster</i> , <i>A. thaliana</i> , <i>C. elegans</i> , <i>P. falciparum</i>
Alternative names	End2p	-
first reference	(Hamburger <i>et al.</i> , 1995)	(Benghezal <i>et al.</i> , 1996)
additional primary references	(Ohishi <i>et al.</i> , 2000; Hiroi <i>et al.</i> , 1998b; Hiroi <i>et al.</i> , 2000)	(Meyer <i>et al.</i> , 2000; Ohishi <i>et al.</i> , 2000; Yu <i>et al.</i> , 1997; Hilley <i>et al.</i> , 2000)
SWISS-PROT accession	P39012	P49018

<sup>#</sup> Additional orthologs were identified by BLAST searches with yeast protein sequences at NCBI (17.09.2000; cf. chapter 7 for www sites).

#### 1.4.4.5. Gpi8p

Gpi8 stands for GPI deficient mutant 8 and was identified in a screen for *S. cerevisiae* GPI biosynthetic mutants being unable to present the GPI-anchored yeast cell wall protein  $\alpha$ -agglutinin at their surface (Benghezal *et al.*, 1995). The *gpi8-1* mutant, as the *end2/gaa1* mutant, accumulates the complete GPI lipid CP2 and an immature 105 kDa form of Gas1p, which, when not anchored by a GPI anchor, cannot leave the ER (Nuoffer *et al.*, 1993). Its temperature-sensitive (ts) phenotype for reduced growth is much less pronounced than that of *end2/gaa1*. To facilitate the selection of complementing genes, the *GPI8* gene was cloned by complementation of a *gpi7-1/gpi8-1* double mutant displaying a strong ts phenotype for growth (Benghezal *et al.*, 1995). Like *GAA1*, *GPI8* is an essential gene. It encodes a type I ER membrane protein having 25 to 28% homology to several plant and invertebrate proteases, which have been classified as the C13 cysteine protease family in the SWISS-PROT database (Benghezal *et al.*, 1995; chapter 7). The C13 family now falls into two subfamilies of more closely related genes, namely the original C13 family and the *GPI8* family; the latter comprises the experimentally verified or predicted orthologs (Table 3) in *Homo sapiens*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Caenorhabditis elegans* showing about 50-60 %, and in *Leishmania mexicana* and *Plasmodium falciparum* showing about 35-40 % of sequence identity over the first 300 amino acid residues with the *Saccharomyces cerevisiae* Gpi8p (Meyer *et al.*, 2000; chapter 3.1.1. and data not shown). The luminal region lacking the transmembrane domain is dispensable for activity as shown for the human Gpi8p (Ohishi *et al.*, 2000).

Although not yet proven, most evidence clearly favors the hypothesis that Gpi8p is the putative catalytic subunit of the GPI:protein transamidase: first, the homology with cysteine proteases (C13 family members), one of which, a jack bean asparaginyl endopeptidase, displayed transamidase activity *in vitro* (Abe *et al.*, 1993), suggests that Gpi8p is directly involved in the proteolytic removal of the GPI-SS; second, the active site of yeast and human Gpi8p consisting of a cysteine (C199 and C206) and a histidine residue (H157 and H164, respectively) was biochemically determined by site-directed mutagenesis based on sequence comparison with C13 cysteine protease members (Meyer *et al.*, 2000; Ohishi *et al.*, 2000) and found to be similar with the structurally and biochemically verified active site of caspases, a related cysteine protease family named C14 in the SWISS-PROT peptidase database. The similarity to the sequence around the active site residues of caspases is more pronounced for Gpi8ps than for the other C13 family members. Active site mutants of yeast Gpi8p are non-functional and display a dominant-negative phenotype when overexpressed in wt cells (Meyer *et al.*, 2000; chapter 3.1.). Thus, there exists biochemical evidence which shows that Gpi8p is related to cysteine proteases suggesting that it might act in a peptidase-like reaction. Third, human Gpi8p has been shown to be required for the formation of the carbonyl intermediate, together with Gaa1p (Ohishi *et al.*, 2000).

However, direct binding of a GPI precursor protein by Gpi8p was identified neither as a carbonyl intermediate nor as another type of interaction. Furthermore, crystal structures of Gpi8p eventually together with substrates, substrate analogs and/or Gaa1p would help to elicit the role of Gpi8p in GPI anchor attachment. Evidence for the role of Gpi8p as transamidase could also come from a demonstration of a proteolytic activity under certain conditions. Up to now, proteolytic activity of Gpi8p was observed neither in an *in vitro* system with purified yeast Gpi8p and synthetic

GPI-SS peptides (Benghezal *et al.*, 1996) nor in wt cells expressing yeast Gpi8p mutants generated by random PCR mutagenesis (chapter 3.2.). Finally, it is considered that establishing an *in vitro* reconstitution system for GPI anchor attachment with purified components might be difficult since it is assumed that not all requirements for GPI anchor addition are known so far (Meyer *et al.*, 2000; Ohishi *et al.*, 2000). However, such a reconstitution system might considerably help to understand the process of GPI anchor attachment and the roles of components of the GPI:protein transamidase therein.

#### 1.4.4.6. Other components of the GPI:protein transamidase

It is not known whether the GPI:protein transamidase contains subunits in addition to Gaa1 and Gpi8 (Ohishi *et al.*, 2000). However, by analogy with the hetero-oligomeric oligosaccharyl transferase complex (OST; Knauer and Lehle, 1999; Karaoglu *et al.*, 1997; Yan *et al.*, 1999; Chi *et al.*, 1996; Reiss *et al.*, 1997) required for N glycosylation, one might also suspect the presence of additional components in the GPI:protein transamidase complex. Ohishi *et al.* (2000) hypothesize that a third component might be present in the GPI: protein transamidase complex that recognizes and presents the preformed GPI, a role that might also be fulfilled by Gaa1p. In addition, the authors hypothesize that a third subunit might bind to an essential region of hGpi8p (residues 311 to 321) which is not required for the interaction with Gaa1p and, because it is not conserved, is also supposed not to be involved in the catalytic reaction. Additional components of the GPI:protein transamidase complex could be identified by means of a classical genetic screen for mutants being synthetically lethal with *gaa1-1* or *gpi8-1*, the latter mutant being less appropriate because of its weak ts phenotype (chapter 3.5.). A biochemical approach, including purification of the protein complex containing Gaa1p and Gpi8p and identification of binding proteins by microsequencing or immunoblotting, could also lead to the discovery of additional transamidase components.

### 1.5. Transport of GPI proteins through the secretory pathway

#### 1.5.1. Transport of GPI proteins through the secretory pathway

Intracellular transport of GPI proteins in general is thought to be similar to that of classical secretory proteins which are transported from the ER to the plasma membrane, i.e. their transport is mediated by vesicular transport between the ER and the Golgi and between the Golgi and the plasma membrane (for reviews see Rothman and Wieland, 1996; Le Borgne and Hoflack, 1998; Kaiser and Ferro-Novick, 1998; Zerial, 1998). It is still unknown how transport through the Golgi really occurs. Three general models could explain the transport between the different Golgi compartments (Rothman and Wieland, 1996; Sciaky *et al.*, 1997; Warren and Malhotra, 1998;): first, a “cargo-forward” model where each passage from a subcompartment to the other is mediated by vesicular traffic including cargo selection, vesicle budding, docking and fusion with the target membrane; second, the Golgi maturation model, i.e. the *cis*-Golgi compartment matures to the *medial*-Golgi subcompartment, the *medial*-Golgi to

the *trans*-Golgi compartment, the *trans*-Golgi to the *trans*-Golgi network (TGN), maturation is insured by vesicular traffic bringing the Golgi-resident enzymes from more mature to more immature stacks; third, traffic between subcompartments is mediated by intermediate vesicular tubules which transiently connect two Golgi subcompartments. During transport through the Golgi apparatus, carbohydrates of GPI proteins are processed. Intracellular transport of GPI proteins has been recently reviewed (Muniz and Riezman, 2000). There is some evidence that GPI-anchored proteins are clustered in sphingolipid-sterol microdomains or rafts. It was suggested that the primary elements of these microdomain components are synthesized mainly in the endoplasmic reticulum. Therefore, it is possible that microdomain assembly starts in the ER and that rafts become larger during transport along the exocytic route. A sorting mechanism for GPI-anchored proteins using sphingolipid microdomains as selective platforms for vesicle budding has been proposed to operate at different steps in the secretory pathway. Furthermore, Ret1p a subunit of COPI coated vesicle have been shown to be required for the forward transport of GPI-anchored proteins from the ER to the Golgi apparatus (Sütterlin *et al.*, 1997).

### 1.5.2. GPI remodeling

GPI lipid remodeling occurs after the addition of the complete GPI precursor glycolipid to immature GPI proteins and remodelase activity has been identified and subcellularly localized to the ER and the Golgi apparatus (Sipos *et al.*, 1997). Two very different types of lipid moieties can be found in yeast GPI anchors: ceramide (Cer) and diacylglycerol (DAG). The ceramides are found on the majority of yeast anchors and consist of mainly C18:0 phytosphingosine (PHS) and a C26:0 fatty acid. Proteins reaching the Golgi can have ceramides with hydroxylated C26:0. A smaller part of yeast GPI proteins including Gas1p contain diacylglycerol with a C26:0 fatty acid (Fankhauser *et al.*, 1993). In both types of lipid moieties, diacylglycerol and ceramide, the C26:0 fatty acid may be hydroxylated on C2.

Since complete GPI lipid precursors lack ceramide and C26:0 in their lipid moiety (generally, they contain diacylglycerol with C16 and C18 fatty acids), it is assumed that these lipids have to be introduced at a stage occurring after GPI anchor attachment by lipid remodeling of protein anchors. Thus, at least three different types of GPI remodelling can be proposed: a) substitution of diacylglycerol by ceramide, b) acyl group exchange on diacylglycerol (DAG) or exchange of DAG for a different DAG, and c) acyl group exchange on Cer or exchange of Cer for Cer or hydroxylation of fatty acids of an anchor Cer. Of course, introduction of very long chain fatty acids (C26) can involve several different exchange reactions and acyl chain hydroxylation. In addition, it has been shown that remodeling of GPI anchors and biosynthesis of inositol phosphoceramides are mediated by different enzymes suggesting that GPI-specific remodelases exist (Reggiori and Conzelmann, 1998).

## 1.6. Extracellular fate of GPI proteins

### 1.6.1. Enzymatic release of GPI proteins

Upon arrival in the extracellular leaflet of the plasma membrane, the GPI anchor becomes a potential substrate for enzymatic release. GPI-anchored proteins were described to be released by PI- or GPI-specific phospholipases C and by GPI-specific phospholipases D. PI-PLCs and GPI-PLD have been isolated from several mammalian tissues, however, GPI-PLD activity and not PI-PLC is present in the mammalian plasma. PI-PLC is thought to be intracellular and soluble, and integral plasma membrane forms were described with the catalytic site on the cytosolic side. GPI-PLD is most probably amphipathic, and is eventually associated with membranes. Recent findings suggest the presence of GPI-PLD activity in lysosomes (for reviews see Nosjean *et al.*, 1997; and references therein).

### 1.6.2. Covalent integration into the yeast cell wall

In addition to the plasma membrane and release into the extracellular medium, yeast cells have a third possibility for the subcellular localization of GPI proteins (cf. also chapter 1.4.2.4), the cell wall. Many cell wall proteins are GPI-anchored and are covalently integrated into the cell wall (most probably by a transamidation reaction). The proteins cannot be removed by simple SDS washes, but need to be released by the action of  $\beta$  glucanases (Lu *et al.*, 1995a; Lu *et al.*, 1994).

## 1.7. Physiological functions of GPI proteins and free GPIs

### 1.7.1. Role of GPI proteins

GPI-anchored proteins play a crucial role in cell metabolism and provide a wide variety of different functions, e.g. as immunoprotective parasitic surface coat proteins, yeast cell wall proteins, mammalian receptors, cell adhesion molecules, differentiation antigens and enzymes (McConville and Ferguson, 1993; Ferguson, 1999). Although several particular functions have been proposed for GPI anchors, including intracellular sorting (Arreaza and Brown, 1995), transmembrane signaling (Brodbeck, 1998), and potocytosis (Anderson, 1998), their most fundamental function consists in the attachment of proteins to the outer leaflet of the plasma membrane in an efficient and stable manner, comparable with a transmembrane polypeptide domain. However, GPI protein anchors differ from transmembrane anchors helices in several respects – such as lateral mobility (Chan *et al.*, 1991; Jacobson *et al.*, 1997), sensitivity to extracellular lipases (Low and Finean, 1977; Guther *et al.*, 1994; Metz *et al.*, 1994), physical isolation from the cytoplasm and the cytoskeleton, and intercellular protein transfer (Anderson *et al.*, 1996), the last property promising a solution for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) patients having a defect in the expression of GPI-anchored proteins (Sloand *et al.*, 1998) and for anti-cancer therapy (McHugh *et al.*, 1999).



### 1.7.2. Role of free GPI glycolipids

In addition to acting as membrane anchors for proteins, the GPI moiety may also regulate processes such as intracellular trafficking and the spatial organization of GPI proteins in the membrane bilayer (Schroeder *et al.*, 1998). Nonprotein-linked, or “free” GPIs, which are either structurally distinct from the protein anchors or present in high excess can also be abundant membrane components in many eucaryotes (Sevlever *et al.*, 1995; Singh *et al.*, 1996). Free GPIs are particularly abundant in parasitic protozoa where they are required for invasiveness. They also have been suggested to play a role in intracellular signaling pathways, however very little is known about their function (Mensa-Wilmot *et al.*, 1999; Ilgoutz *et al.*, 1999).

## 2. GOAL OF THE WORK

Benghezal *et al.* (1996) reported the identification of the yeast Gpi8p as being essential for GPI anchor attachment to proteins. The *GPI8* gene was cloned by complementation of the double mutant *gpi7-1 gpi8-1*. The *gpi8-1* was found to be temperature-sensitive for growth and to accumulate the complete GPI precursor glycolipid CP2, and to retard maturation of the yeast GPI model protein Gas1p at 37 °C, a phenotype which was expected for a GPI:protein transamidase mutant. Furthermore, the protein was described to be present in the endoplasmic reticulum, mainly on the luminal side and to have homologies to known plant cysteine proteases. The latter property was a first indication that Gpi8p might be the catalytic subunit of the putative GPI:protein transamidase complex.

The major goal of this thesis was the further characterization of the yeast *Saccharomyces cerevisiae* Gpi8 protein and of its role in the process of GPI anchor attachment. A first aim consisted in the identification of the predicted active site of this protease-like protein. Another challenge was to analyze whether wt or mutant Gpi8p can display a proteolytic activity. Benghezal *et al.* (1996) reported that no proteolytic activity of purified Gpi8p had been observed *in vitro*. Third, the interaction with one of its substrates, the immature GPI-protein, had to be studied in order to answer the question whether Gpi8p alone is required for the specific recognition of the GPI signal sequence which acts as a signal for GPI anchor attachment.

An additional aim of this thesis was to support the ongoing work in the laboratory aiming at the isolation of additional components or subunits of the putative GPI protein transamidase, if they really exist. Gaa1p identified by Hamburger *et al.* (1995) was already supposed to be another candidate for a subunit of the transamidase since its temperature-sensitive mutant showed a GPI anchoring defect comparable with that of *gpi8-1* cells. Additional subunits might be identified by various strategies including genetic screens, co-immunoprecipitation studies and the yeast Two-Hybrid system. Genetic screens were described several times to be a successful method to identify novel genes belonging to the same complex or the same metabolic path, one example being the oligosaccharyl transferase complex required for *N* glycosylation (Reiss *et al.*, 1997; Yan *et al.*, 1999). The selection for synthetic lethals to with known mutant having a strong conditional phenotype is such a classical genetic screen. The mutant *gpi8-1* isolated by Benghezal *et al.* (1995) had only a weak temperature-sensitive phenotype for growth and was therefore considered as not being useful for a synthetic lethality screen. Therefore, we looked for a *gpi8* mutant with a strong temperature-sensitive phenotype for growth using mutagenesis of the wt *GPI8* gene followed by growth analysis.

Furthermore, since the phenotype of GPI lipid remodeling-deficient cells is totally unknown and cannot be predicted easily, it is nearly impossible to plan a simple selection procedure to identify GPI remodeling mutants. Therefore, the whole yeast genome was screened for possible candidates for GPI lipid remodelases by performing computational homology searches.

### **3. RESULTS**

#### **3.1. Active site determination of Gpi8p, a Caspase-related enzyme Required for Glycosylphosphatidylinositol Anchor addition to pro- teins**

##### **3.1.1. Publication**

## Active Site Determination of Gpi8p, a Caspase-Related Enzyme Required for Glycosylphosphatidylinositol Anchor Addition to Proteins<sup>†</sup>

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**ABSTRACT:** Glycosylphosphatidylinositol (GPI) anchors are attached to newly synthesized proteins in the ER by a transamidation reaction during which a C-terminal GPI attachment signal is replaced by a preformed GPI precursor lipid. This reaction depends on *GAA1* and *GPI8*, the latter belonging to a novel cysteine protease family. Homologies between this family and other Cys proteinases, such as caspases, pointed to Cys199 and His157 as potential active site residues. Indeed, *gpi8* alleles mutated at Cys199 or His157 are nonfunctional, i.e., they are unable to suppress the lethality of  $\Delta gpi8$  mutants. The overexpression of these nonfunctional alleles in wild-type cells leads to the accumulation of the free GPI precursor lipid CP2, delays the maturation of the GPI protein Gas1p, and arrests cell growth. The dominant negative effect of the Cys199 mutant cannot be overcome by the simultaneous overexpression of Gaa1p. Most *GPI8* alleles mutated in other conserved regions of the protein can complement the growth defect of  $\Delta gpi8$ , but nevertheless accumulate CP2. CP2 accumulation, a delay in Gas1p maturation and a slowing of cell growth can also be observed when Gpi8p is depleted to 50% of its normal level in wild-type cells. The dominant negative effect of nonfunctional and partially functional mutant alleles can best be explained by assuming that Gpi8p works as part of a homo- or heteropolymeric complex.

As in all other eucaryotes, the yeast GPI<sup>1</sup> anchor is attached to newly translocated proteins in the ER by a process in which a C-terminal hydrophobic GPI anchoring signal sequence is removed and a preformed GPI is attached in its place (1–4). The GPI transferase is believed to act as a transamidase, i.e., to simultaneously remove the GPI anchoring signal and to replace it with the preformed GPI. Thus, at least in living cells, one cannot observe biosynthetic intermediates from which the hydrophobic GPI signal has been removed, but to which a GPI has not yet been added. This holds true not only for normal cells but also for mammalian mutant cell lines unable to synthesize complete GPIs (5, 6). Recently, evidence for a transamidase has also been inferred from the finding that a microsomal enzyme activity capable of removing the C-terminal GPI anchor signal is enhanced by small nucleophilic amines (7). Genetic approaches have identified genes required for addition of GPI precursor lipids to proteins. Transamidase-deficient cells are expected to accumulate complete GPI lipids as well as GPI precursor proteins. This phenotype is exhibited by two yeast mutants, *gaa1* and *gpi8*, and a mammalian mutant cell line (class K) (8–11). The *GAA1* gene is essential and

encodes a 68 kD ER protein with a large luminal domain, several membrane spanning domains, and a cytosolic ER retrieval signal on its extreme C-terminus. The exact role of Gaa1p has not yet been elucidated.

*GPI8* is also an essential gene and encodes a type I ER membrane protein having 25–28% homology to several plant and invertebrate proteases, which have been classified as cysteine proteinase family C13 in the SWISSPROT database (12–15). By homology searches, we recently identified three additional sequences, which have high homology to yeast and human *GPI8* (*yGPI8* and *hGPI8*) and that seem to represent the GPI8 homologues of *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*. Over the first 300 amino acids, their sequence shows 50, 58, and 53% identity and 83, 87, and 81% of similarity with *yGPI8*, respectively. *yGPI8* has been associated with the C13 protease family of the SWISSPROT protein sequence database, which, as a result of this, now falls into two subfamilies of more closely related genes, namely, the original C13 family and the *GPI8* family presently comprising 5 *GPI8* homologues (Figure 1a and 1b). The homology with proteases does not prove, but at least suggests that Gpi8p is directly involved in the proteolytic removal of the GPI-anchoring signal. If such were the case, then the mutation of the predicted active site residues should produce nonfunctional alleles of *GPI8*. Only one single Cys is conserved within the original C13 subfamily and has been proposed to be the active site Cys of the original C13 family (16). This Cys is not conserved in *yGPI8*, but next to it there is a Ser (Ser60, Figure 1b). Since it has been demonstrated experimentally for some cysteine proteases that some proteolytic activity is still preserved when the active site Cys is changed

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<sup>1</sup> Abbreviations: BCS, bathocuproinedisulfonic acid; CP, complete precursor; FOA, 5-fluoroorotic acid; GPI, glycosylphosphatidylinositol; Ins, *myo*-inositol; wt, wild-type.

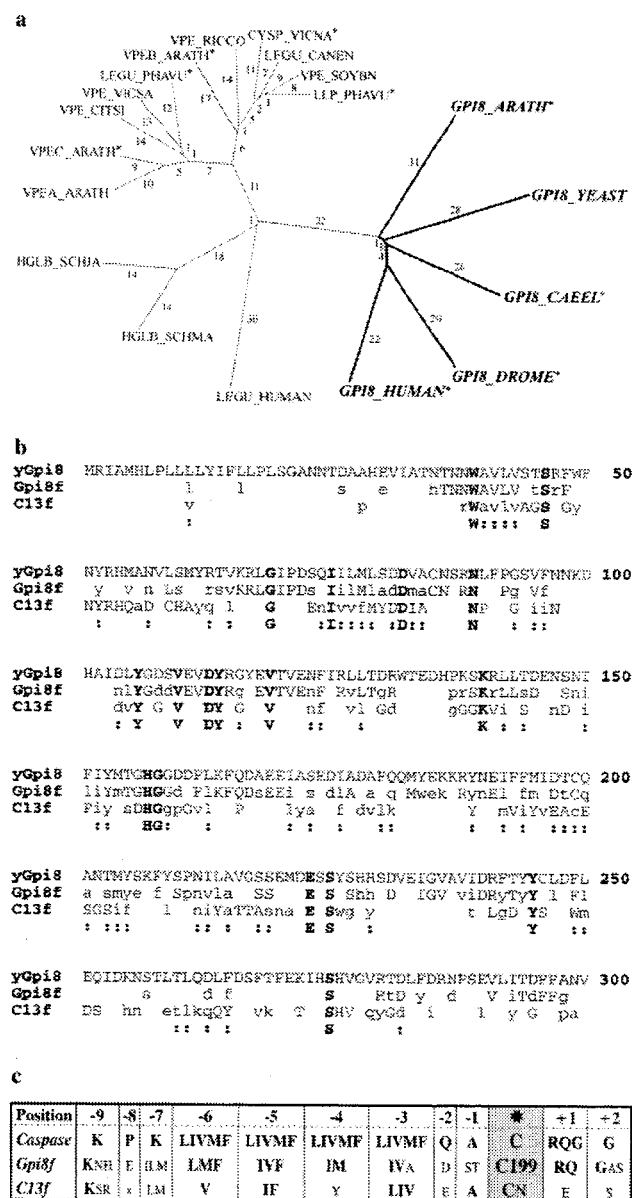


FIGURE 1: Relationship of *GPI8* with other Cysteine proteases. Panel a: The phylogenetic tree of the SWISSPROT C13 cysteine protease family and its newly identified homologues was established by a ClustalW alignment and visualized by TreeView (57). Numbers correspond to the phylogenetic distance calculated with the neighbor-joining method (58) and multiplied by 100. Some proteins (\*) are not yet classified in the SWISSPROT database but were named following the SWISSPROT nomenclature; their TrEMBL accession numbers are O46047 (*GPI8\_DROME*), O14822 (*GPI8\_HUMAN*), O64674 (*GPI8\_ARATH*), Q39044 (*VPEB\_ARATH*), O24325 (*LEGU\_PHAVU*), Q39119 (*VPEC\_ARATH*), O24326 (*LLP\_PHAVU*), and O24539 (*CYSP\_VICNA*). The SWISSPROT accession number of *GPI8\_CAEL* is P49048. Panel b: Sequence alignment of the first 300 amino acids of *S. cerevisiae* Gpi8p with the consensus sequence of the Gpi8p subfamily (Gpi8f) and the original C13 Cys protease family, excluding the Gpi8p subfamily members (C13f). Capital letters define absolutely conserved amino acid residues, small letters represent residues that are conserved in more than 50% at sites sharing similar physicochemical properties. The bottom line gives an overall consensus sequence for the C13 Cys protease family; positions where residues have similar physicochemical properties are marked by colons. Panel c: Alignment of the PROSITE pattern of the active site cysteine residue of caspases (PS01122) with the regions around the proposed active site Cys of the *GPI8* (Gpi8f) and the original C13 Cys protease subfamilies (C13f). Identical amino acid residues are highlighted in bold.

to Ser (17–21), we initially considered the possibility that Ser60 may be the active site. Therefore, Ser60 was mutated to Ala and also to Cys and the whole region containing Ser60 of *yGPI8* (residues 55–61) was replaced by the corresponding conserved consensus sequence, which is present in the other members of the C13 family. This consensus sequence contains a Cys in position 59 and the mutant is called S60—C59. When it became clear that the S60A mutation was still functional, Cys residues were mutated. Of the four Cys residues of *yGPI8* only two (Cys85 and Cys199 of *yGPI8*) are conserved in all five-members of the *GPI8* subfamily. None of them is located within one of the many conserved blocks of sequence identity (Figure 1b). Interestingly, Cys199 is also conserved in all but one member of the original C13 protease family, the exception being the *Schistosoma mansoni* hemoglobinase B. Cys85, on the other hand, is not at all conserved in the rest of the C13 protease family. Cys199 was strongly suggested as the active site residue by sequence comparison of the C13 protease family with the C14 Cys protease family, i.e., the caspase family, which seems to be the most closely related Cys protease family. The X-ray structures of two caspases have been reported and their active site residues have been determined (22). As shown in Figure 1c, the region around the Cys199 of *yGPI8* bears a distinct resemblance with the PROSITE motif found around the active site Cys in the caspase family (PROSITE pattern PS01122).

The study of Cys proteases showed that many of them contain a catalytic triad of Asn, His, and Cys (papain, PROSITE document PDOC00126) or at least a catalytic diad of His and Cys (caspases, PDOC00864), whereby His helps to deprotonate the active site Cys in the same way as is described for the active site Ser of serine proteases (23, 24). We, therefore, mutated His157, which is conserved in the original C13 as well as the *GPI8* family (Figure 1b). We also mutated His54 of *yGPI8*, which is conserved in 18 of the 19 C13 protease family members, the exception being *A. thaliana GPI8* (*atGPI8*), which, however, contains a His nearby.

## MATERIALS AND METHODS

**Strains, Media, and Materials.** *Saccharomyces* strains are listed in Table 2A and were grown in minimal medium supplemented with all 20 amino acids (20–400 mg/L), adenine sulfate, and uracil (SDaa medium). SGaa medium is SDaa medium with 2% galactose instead of glucose. The copper concentration of SDaa media was determined with the copper chelating agent BCS (25) and amounted to 0.75–1.0  $\mu$ M. Copper-free media contained 100  $\mu$ M BCS and 1  $\mu$ M FeCl<sub>2</sub>. The absorbance of dilute cell suspensions was measured at 600 nm, one OD<sub>600</sub> unit of cells corresponding to  $1\text{--}2.5 \times 10^7$  cells. Alternatively, cells were counted microscopically. Reagents were purchased from the following sources: Bathocuproinedisulfonic acid disodium salt (BCS) from Fluka; [2-<sup>3</sup>H]-*myo*-inositol, 20 Ci/mmol from Anawa; [<sup>35</sup>S]-methionine/[<sup>35</sup>S]-cysteine Prot. Laboratory Mix, Moravick; anti-mouse and anti-rabbit IgG—peroxidase conjugates from Sigma. Polyclonal rabbit antibodies against CPY were raised as described (26). Antibodies against Cwp1 and Yap3p were kindly donated by Dr. H. Shimoi (National Research Institute of Brewing, Kagamiyama, Japan) and Dr. Y. Bourbonnais (University Laval, Québec, Canada), respectively. Gaalp-

Table 1: Complementation of the Growth Defect of  $\Delta gpi8^a$ 

transfected <i>gpi8</i> allele	tetrad analysis					plasmid shuffling	
	total of tetrads	number of viable spores/tetrad					clones growing on FOA(%)
		0	1	2	3	4	
empty vector	9	1	1	7		0	
<i>GPI8</i>	9		1	1	2	5	100
H54A	23		1	5	5	12	100
S60A	17	1	1	5	2	8	30
S60C	18	2	2	4	6	4	100
S60→C59	17		4	13			0
C85A							29
H157A	18	3	4	11			0
C199A	18	3	4	11			0

<sup>a</sup> FBY143 ( $\Delta gpi8/GPI8$ ) harboring multicopy YEp*GPI8*-type plasmids with various *GPI8* alleles under the control of the *GPI8* promoter were sporulated and tetrads were dissected. The number of tetrads yielding 4, 3, 2, 1, or no growing spores are indicated in bold. In all tetrads giving four colonies only two grew in the presence of FOA (not shown). Plasmids were recovered from 2 to 4 independent FOA-sensitive, geneticin-resistant colonies, harboring either S60A, S60C, or H54A. On all of these plasmids harboring S60A and S60C, the first 500 bases of *GPI8* were sequenced in order to exclude reversions/second site mutations. H54A containing plasmids were verified by digestion with *NcoI*. For plasmid shuffling (last column), the haploid strain FBY525 ( $\Delta gpi8/YEpGPI8$ ) was transfected with the multicopy vector pBF55 for C85A or the single copy vector pBF53 harboring various alleles of *GPI8*; 12–40 clones from each transfected strain were streaked out on FOA containing plates to see if the wt *GPI8* (on YEp*GPI8*) could be forced out. The percentage of clones giving viable progeny on FOA is indicated. Plasmids were recovered from FOA-resistant colonies harboring either a S60A, S60C, or H54A mutation and verified by sequencing as above.

overexpressing plasmids, pDH15 and pDH17, were kindly provided by Dr. Howard Riezman (Biocenter, Basel, Switzerland), pGGA1, a plasmid containing a guar  $\alpha$ -galactosidase/ $\alpha$ -agglutinin under the *GAL7* promoter was the kind gift of Dr. Marteen P. Schreuder (University of Amsterdam, Netherlands), and the plasmid pFBY166, harboring the *CUP1* promoter was contributed by Dr. Jutta Heim (Novartis, Basel, Switzerland). The production of anti-Gas1p (27) and of affinity purified anti-Gpi8p rabbit antibodies (28) has been described.

**Construction of *gpi8* Mutant Alleles.** Plasmids used in this study are listed in Table 2B and were constructed using standard procedures (29). Point mutations were introduced into the *GPI8* gene by means of artificial DNA fragments; partially overlapping, synthetic oligonucleotides were annealed, gaps were filled by bacteriophage T4 DNA polymerase and the resulting DNA fragments were digested with restriction enzymes before being ligated into the plasmid YEp*GPI8*. The inserted sequences of all constructs were verified by sequencing. The following residues of y*GPI8* were replaced by substituting with Ala using codon GCC: His54 (H54A), Ser60 (S60A), His157 (H157A), and Cys199 (C199A). Cys85 was replaced by Ala using codon GCG (C85A). Ser60 was also replaced by cysteine (TGT; S60C); in addition, the amino acid residues MANVLSM (positions 55–61) were replaced by the C13 cysteine protease consensus sequence QADVCHA (CAAGCTGATGTCTGT-CACGCT; S60→C59). To express mutant alleles from a single copy vector, the 2374 bp *SstI/SalI* fragment of YEp*GPI8*, harboring the complete *GPI8* gene, was introduced into the *SstI/SalI* sites of YCplac22, resulting in plasmid pBF53. Mutant alleles were generated in this vector

by excising the 2286 bp *XhoI/XbaI* fragment of pBF53 and replacing it with the 2286 bp *XhoI/XbaI* fragments of YEp*GPI8* plasmids containing mutant alleles.

To generate *gpi8* alleles under the control of the *GAL1-10* promoter, a *BamHI* restriction site was introduced by PCR upstream of the ATG of *GPI8* by using the primers *GPI8*-for*Bam* (5'-AAGCCGGGATCCATGCGTATAGCGATG-3') and *GPI8*-rev400 (5'-AAAAGTCTCGAGATTAGTGTA-CAGGTC-3'). The PCR fragment was digested with *BamHI* and *SallI*; the resulting 79 bp fragment was cloned together with the 1629 bp *AlwNI/SalI* fragment of YEp*GPI8* into the vector YIpGal (30) digested with *SstI* and *XhoI* to yield the plasmid YIpGAL*GPI8*. Before transfection YIpGAL*GPI8* was digested with *StuI* in order to direct its integration into the *ura3-1* locus of W303-1B or FBY143. After transformation of FBY143 with YIpGAL*GPI8*, *ura*<sup>+</sup> transformants were sporulated and tetrads were dissected and germinated on rich galactose medium.  $\Delta gpi8/GAL1-10-GPI8$  strains such as FBY164 were obtained by selecting geneticin-resistant uracil prototrophs.

The *GPI8* gene was fused with the *CUP1* promoter by first linearizing the plasmids pFBY166 (obtained through Dr. J. Heim, Novartis, Basel; (31)) and YIpGAL*GPI8* with *EcoRI* and *NciI*, respectively. The plasmids were treated with the Klenow fragment of *Escherichia coli* DNA polymerase I in order to produce blunt ends and were then digested with *SalI*. Ligation of the 3752 fragment of pFBY166 and the 1712 bp fragment of YIpGAL*GPI8* produced the plasmid pBF58. The 2335 bp *SphI/SalI* *CUP1-GPI8* fragment of pBF58 was then introduced into the *SphI/SalI* sites of the YCplac22 and YEplac112, yielding the plasmids pBF54 and pBF55, respectively. Plasmids harboring mutant alleles of *GPI8* under the control of the *CUP1* promoter were created by replacing fragments of pBF54 and pBF55 by the corresponding mutant fragments excised from YEp*GPI8*-type plasmids. Yeast strains were transformed by electroporation (32).

**Protein Extraction and Western Blot Analysis.** Cells were broken by vortexing with glass beads in TEPI buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% dimethyl sulfoxide (DMSO), 2 mM phenylmethylsulfonyl fluoride (PMSF) and antipain, leupeptin and pepstatin, each at 30  $\mu$ g/mL; 20  $\mu$ L per OD<sub>600</sub> of cells). After the addition of 5-fold concentrated reducing sample buffer, extracts were denatured during 5 min at 95 °C and separated by SDS-PAGE (33). Western blots were developed by enhanced chemiluminescence (ECL Kit, Amersham, Buckinghamshire, UK). Expression of Gpi8p was quantitated by densitometry using a Bio-Rad Imaging Densitometer, Model GS-700, and the Molecular Analyst 2.1 software (Bio-Rad Laboratories, Glattbrugg, Switzerland).

**Radiolabeling of GPI Lipids and GPI Proteins.** Cells were preincubated for 10 min and labeled with [2-<sup>3</sup>H]myo-inositol (2  $\mu$ Ci/OD<sub>600</sub> of cells) for 60 min at 37 °C as described (10). Lipids were extracted with chloroform/methanol/water 10:10:3 (v/v/v) and desalted by butanol/water phase separation as described (34). Lipid extracts were analyzed by ascending TLC using 0.2 mm-thick silica gel 60 plates with the solvent system chloroform/methanol/water 10:10:3. [2-<sup>3</sup>H]-myo-inositol- and [2-<sup>3</sup>H]-mannose-labeled standards were produced as described previously (34). Radioactivity was detected by one- and two-dimensional radioscanning and fluorography. Maturation of Gas1p and CPY was analyzed by pulse-chase labeling experiments at 37 °C with [<sup>35</sup>S]methionine and [<sup>35</sup>S]-

Table 2

(A) Strains Used in this Study		
strain	genotype	plasmid
W303-1B	<i>MAT<math>\alpha</math> ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15</i>	
FBY11	<i>MAT<math>\alpha</math> ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1</i>	
FBY122	<i>MAT<math>\alpha</math> ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 gpi7-1 gpi8-1</i>	
FBY164	<i>MAT<math>\alpha</math> ade2-1 leu2-3,112 his3-11,15 <math>\Delta</math><i>gpi8::kanMX2</i> ura3-1::URA3-GAL1-10-GPI8</i>	
FBY525	<i>MAT<math>\alpha</math> ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 lys<sup>-</sup> <math>\Delta</math><i>gpi8::kanMX2</i></i>	YE <i>pGPI8</i>
FBY526	FBY525	pBF53
FBY577	W303-1B; <i>ura3-1::URA3-GAL1-10-GPI8-myc-his6</i>	
FBY578	W303-1B; <i>ura3-1::URA3-GAL1-10-GPI8_C199A-myc-his6</i>	
FBY554	FBY525	pBF53_H54A <sup>a</sup>
FBY555	FBY525	pBF53_S60A <sup>a</sup>
FBY558	FBY525	pBF53_S60C <sup>a</sup>
FBY143	<i>Mata/<math>\alpha</math> ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 TRP1-1/trp1-1 lys<sup>+</sup>/lys-GPI8/<math>\Delta</math><i>gpi8::kanMX2</i></i>	
(B) Plasmids Used in This Study		
plasmid	description	reference
YC <i>plac22</i>	<i>TRP1 CEN4-ARS1 Ap<sup>R</sup></i>	(55)
YE <i>plac112</i>	<i>TRP1 2<math>\mu</math> Ap<sup>R</sup></i>	(55)
YE <i>plac195</i>	<i>URA3 2<math>\mu</math> Ap<sup>R</sup></i>	(55)
YE <i>p352</i>	<i>URA3 2<math>\mu</math> Ap<sup>R</sup></i>	(56)
YE <i>pGPI8</i>	YE <i>p352</i> ; <i>GPI8</i>	(28)
Y <i>pGAL</i>	<i>URA3 Ap<sup>R</sup></i>	(30)
Y <i>pGALGPI8</i>	Y <i>pGAL</i> ; <i>GAL1-10-GPI8</i>	this study
pBF53	YC <i>plac22</i> ; <i>GPI8</i>	this study
pBF54	YC <i>plac22</i> ; <i>CUP1-GPI8</i>	this study
pBF55	YE <i>plac112</i> ; <i>CUP1-GPI8</i>	this study
pDH15	YE <i>plac195</i> ; <i>GAA1</i>	(9)
pDH17	YE <i>plac112</i> ; <i>GAA1</i>	(9)

<sup>a</sup> For plasmids which harbor mutant alleles of GPI8, the type of mutation is indicated after the plasmid name.

cysteine (20  $\mu$ Ci/OD<sub>600</sub>) followed by immunoprecipitation using polyclonal rabbit antisera and protein A-Sepharose as described previously (35).

## RESULTS

**Assessment of Functionality of GPI8 Alleles through Complementation of the Growth Defects of  $\Delta$ *gpi8* and *gpi7-gpi8*.** Mutant alleles of *GPI8* were expressed in a  $\Delta$ *gpi8*/*GPI8* heterozygote diploid strain, and the viability of the  $\Delta$ *gpi8* progeny was assessed by tetrad analysis. As shown in Table 1, diploids containing the empty vector only yielded two viable colonies per tetrad and the wild-type (wt) *GPI8* rescued the growth of the  $\Delta$ *gpi8* colonies. Mutations C199A and H157A as well as S60 $\rightarrow$ C59 were completely unable to rescue the  $\Delta$ *gpi8* spores. H54A, S60A, and S60C were still able to complement  $\Delta$ *gpi8* strains although the fraction of tetrads with less than four viable spores seemed to be somewhat higher with these alleles (Table 1).

The *gpi7-1-gpi8-1* double mutants are unable to grow at 37 °C, whereas *gpi7-1* and *gpi8-1* cells grow almost normally at 37 °C (28). Thus, *gpi7-1-gpi8-1* have a more severe GPI-anchoring defect than either single mutant. To detect residual activity of *gpi8* mutant alleles, we transfected them on a multicopy vector into the *gpi7-gpi8* double mutant and assessed the growth of the transfectants at 30, 33, and 37 °C on plates or in liquid cultures. While wt *GPI8* restored normal growth at 37 °C, neither C199A, H157A, nor S60 $\rightarrow$ C59 improved the growth of the double mutant at these temperatures, suggesting that none of these alleles had any residual activity (not shown). Surprisingly, the same result was also obtained when transfecting *gpi7-gpi8* with H54A, S60A, or S60C, which are able to rescue the lethality of

$\Delta$ *gpi8* strains and thus are partially active (for discussion, see below). As can be seen in Table 1, expression of mutated forms of *GPI8* in the  $\Delta$ *gpi8* background using plasmid shuffling gave the same result as dissection of tetrads: C199A, H157A, and S60 $\rightarrow$ C59 were inactive, whereas H54A, S60A, S60C, and C85A complemented the lethal effect of the *GPI8* deletion.

**Overexpression of H157A and C199A Alleles in Wt Cells Leads to Growth Arrest.** If we assume that Gpi8p works as part of a complex with other subunits, then we may expect that the overexpression of nonfunctional *gpi8* alleles will compromise the GPI anchor addition and hence cell growth. To test for such a dominant negative effect, we overexpressed H157A and C199A alleles from a multicopy vector in wt cells. When we used the natural *GPI8* promoter, we could not observe any significant growth retardation in any clone, but when probing the cell extracts with anti-Gpi8p antibodies on Western blots, we realized that H157A and C199A were overexpressed only 3–4-fold over the wt level present in nontransfected cells, whereas wt Gpi8p was overexpressed 9-fold. This phenomenon was observed in several independent experiments, and we had to conclude that the plasmids containing H157A and C199A alleles were selected against or that the nonfunctional Gpi8p proteins were more rapidly degraded.

To obtain stronger overexpression, some *gpi8* mutant alleles were placed on a multicopy vector under the control of the Cu<sup>2+</sup> inducible *CUP1* promoter. After transfection into wt cells and induction for 1 h with 500  $\mu$ M Cu<sup>2+</sup> overexpression of wt Gpi8p was very strong and generated a large array of degradation products and also of higher molecular weight products, which may be ubiquitinated or hypergly-

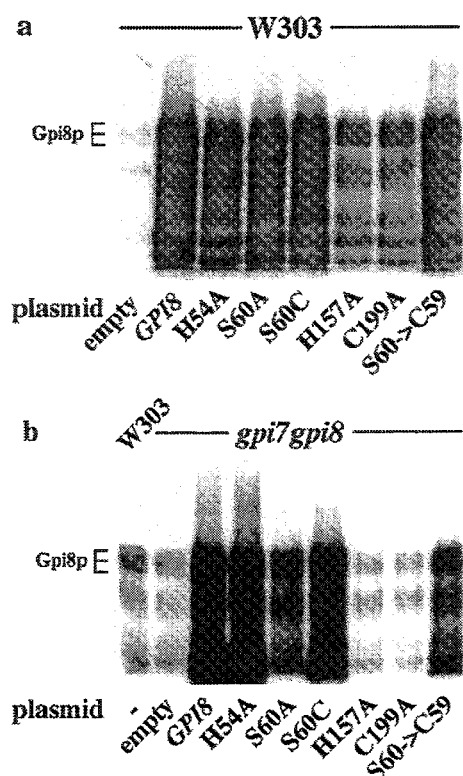


FIGURE 2: Overexpression of mutant Gpi8p in W303 and *gpi7-gpi8*. *gpi8* alleles were expressed under the control of the strong, copper inducible *CUP1* promoter using the multicopy vector pBF55 (Table 2B). Cells were cultured at 24 °C in SDaa (panel a) or copper-free SDaa/BCS (panel b) to exponential phase. One hour before extraction, conditions were changed in that cells were shifted to 37 °C (panel a) and that  $\text{CuSO}_4$  was added to 500  $\mu\text{M}$  (panel a) or 200  $\mu\text{M}$  (panel b). Overexpression of Gpi8p was analyzed by SDS-PAGE followed by Western blotting using affinity purified anti-Gpi8p and was quantified by densitometry.

cosylated forms of Gpi8p (Figure 2a). If for quantification we only take into consideration the bands of 46–50 kD, the transfected wt Gpi8p, C199A, and H157A alleles were overexpressed, respectively, 72, 36, and 23-fold over the physiological, endogenous level of Gpi8p. *CUP1* promoter driven C199A and H157A alleles of GPI8 were much less well-expressed in the *gpi7-gpi8* double mutant. Indeed, *gpi7-gpi8* cells, harboring C199A and H157A, could not be induced to express more Gpi8p than cells containing the empty vector (Figure 2b), probably because of very heavy selection against plasmids carrying  $\text{Cu}^{2+}$ -inducible *GPI8* alleles due to significant basal transcription from the *CUP1* promoter even in the absence of copper and the presence of the copper chelator, BCS. Significantly, S60A, S60C, and S60→C59 were also less well-expressed than wt *GPI8* (Figure 2b).

While the growth of all cells, including nontransfected wt cells, was somewhat diminished in the presence of  $\text{Cu}^{2+}$ , we observed a drastic growth inhibition in cells harboring H157A and C199A (Figure 3a and b), which was particularly pronounced in cells harboring C199A. The same phenomenon was also observed when *CUP1* promoter driven C199A and H157A alleles were expressed from a single copy vector or upon induction of a *GALI-10* promoter driven genomic copy of C199A (data not shown). On the other hand, massive overexpression of S60→C59, H54A, S60A, and S60C from multicopy vectors and under the *CUP1* promoter had no

effect on the growth rate of wt cells (Figure 3a, 3b, and data not shown).

The dominant negative effect of C199A overexpression on cell growth could be counteracted by the concomitant overexpression of wt Gpi8p. As can be seen in Figure 3c, growth repression caused by induction of C199A in the presence of  $\text{Cu}^{2+}$  was suppressed by the concomitant overexpression of wt Gpi8p under the *GALI-10* promoter. In control experiments, we found that the type of hexose used as carbon source (Gal or Glc) had no influence on the growth rate of wt cells in the presence of 500  $\mu\text{M}$   $\text{Cu}^{2+}$  nor on the kinetics by which cells harboring a *CUP1* promoter driven C199A ceased to grow upon addition of  $\text{Cu}^{2+}$  (not shown). Similarly, the growth arrest caused by the induction of *GALI-10* promoter driven C199A was completely reversed by the expression of wt Gpi8p under the control of the *CUP1* promoter (not shown).

For a more sensitive assay, we tried to overexpress *GPI8* alleles in the *gpi7-gpi8* double mutant (Figure 3d). After the mere transfection of C199A and H157A, cells had a severely reduced growth rate even at the permissive temperature (24 °C) and survived only in the presence of the copper chelator, BCS. Removal of BCS further reduced cell growth, and addition of  $\text{Cu}^{2+}$  stopped the cell growth almost immediately (Figure 3d).

**Accumulation of Free GPI Lipids in the Presence of Mutated Gpi8 Proteins.** Figure 3 shows that upon induction, the growth of wt cells, harboring C199A and H157A alleles, stopped or slowed only after 4–8 cell divisions. This can be explained in several ways: (i) The association of Gpi8p with the hypothetical transamidase complex might be very stable so that nonfunctional Gpi8p appearing upon induction with  $\text{Cu}^{2+}$  will not get integrated into preexisting transamidase complexes, but only into new complexes formed from newly synthesized components. Thus, functional complexes may persist for prolonged periods. (ii) Nonfunctional Gpi8p proteins may immediately get access to preexisting transamidase complexes and thus rapidly diminish the cell's capacity to attach GPI anchors to newly made GPI proteins, but wt cells may contain the essential GPI proteins in large excess so that they can go through several rounds of cell division without making new GPI proteins. In an attempt to distinguish these possibilities, we undertook to monitor how fast, upon overexpression of dominant negative *GPI8* alleles in wt cells, substrates of the transamidase would accumulate.

As previously reported, the precursor lipids (CPs) remain undetectable in the lipid extract of [ $^3\text{H}$ ]Ins-labeled wt cells, even when their addition to newly made proteins is interrupted by the addition of cycloheximide (34). W303 wt cells, harboring different mutant alleles of *GPI8* on a multicopy vector under the control of the *CUP1* promoter, were induced for 1 h with  $\text{Cu}^{2+}$ , and while one aliquot of the cells was used to determine the amount of Gpi8p expression as shown in Figure 2a, another aliquot was labeled with [ $^3\text{H}$ ]Ins for 60 min. As can be seen in Figure 4a, all cells expressing mutant alleles began to accumulate CP2 as well as two forms of the immature precursor M4 (36, 37). Accumulation of CP2 was not observed without induction with  $\text{Cu}^{2+}$  (not shown). Significantly, accumulation of CP2 is most pronounced in cells containing C199A and H157A, i.e., the alleles that have the greatest dominant negative effect on cell growth. The same result was also obtained when H157A



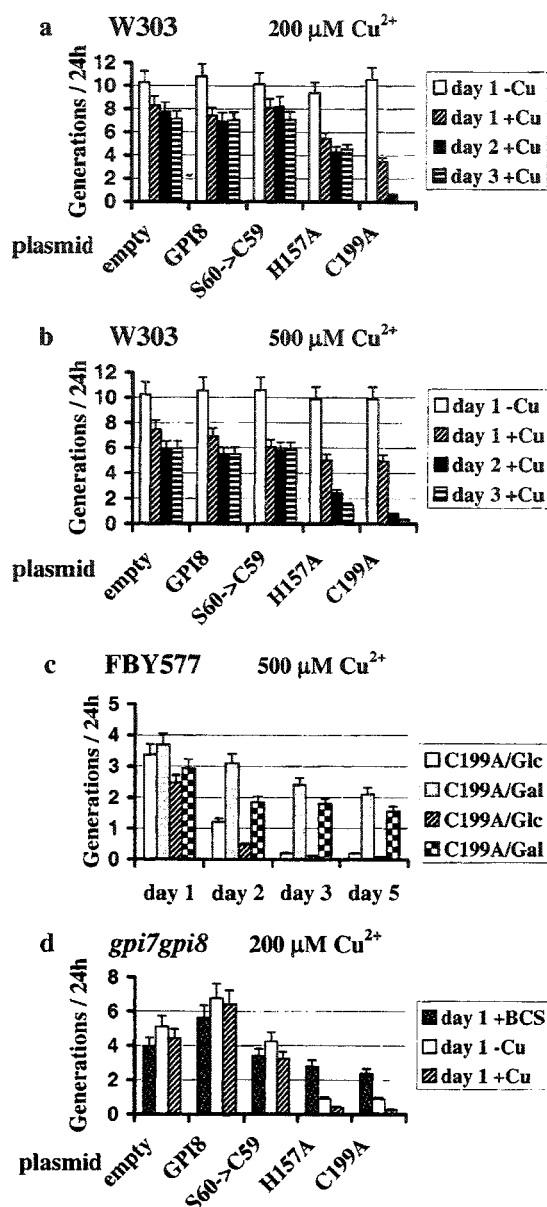


FIGURE 3: Expression of nonfunctional *gpi8* alleles H157A and C199A arrests cell growth of W303 and *gpi7-gpi8*. *gpi8* alleles under the control of the *CUP1* promoter were present on the multicopy or centromeric vectors pBF55 or pBF54, which were retained by omission of trp from growth media. Panels a, b: W303 cells harboring *gpi8* alleles on pBF55, were grown to exponential phase at 24 °C without  $\text{Cu}^{2+}$  (0.75–1  $\mu\text{M}$   $\text{Cu}^{2+}$ ) in SDaa medium and were resuspended at 0.2–0.3 OD<sub>600</sub>/mL in fresh normal (–Cu) or  $\text{CuSO}_4$ -containing (+Cu) SDaa medium. After 1 h at 24 °C, cultures were shifted to 37 °C. Panel c: Exponentially growing FBY577 (W303 cells harboring a genomically inserted copy of wt *GPI8* under the *GALI-10* promoter) and harboring C199A under the *CUP1* promoter on pBF54 (light-shaded, dark-shaded) or pBF55 (slanted line, checked) were resuspended at 0.2–0.3 OD<sub>600</sub>/mL in fresh glucose or galactose medium and kept at 37 °C for 6 h.  $\text{Cu}^{2+}$  was then added to 500  $\mu\text{M}$  to all cultures. Panel d: The temperature-sensitive *gpi7-gpi8* double mutant harboring *gpi8* alleles on pBF55 was grown up in copper-free SDaa medium (100  $\mu\text{M}$  BCS) and then cultured further either in copper-free (BCS), normal (–Cu) or  $\text{CuSO}_4$ -containing (+Cu) medium at the permissive temperature (24 °C). Cell growth was monitored at short intervals during 1–3 days, whereby cells were repeatedly diluted when reaching 2.0 OD<sub>600</sub>/mL. The cell concentrations of triplicate cultures were determined by both densitometry and cell counting. Columns indicate the number of cell generations during successive periods of 24 h.

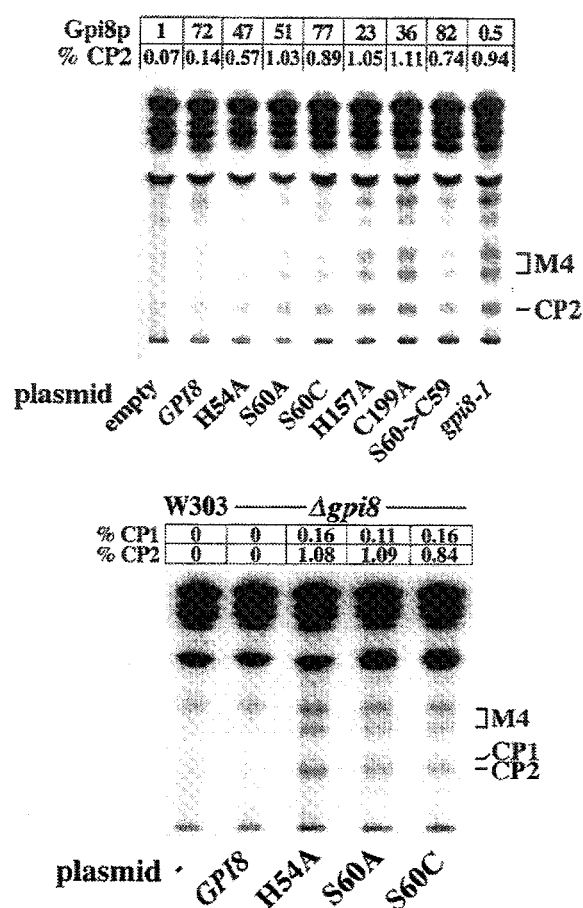


FIGURE 4: Cells harboring various mutant alleles of *Gpi8p* accumulate CP2. Panel a: Data shown here and in Figure 2a were obtained with cells from the very same cultures. W303 wt cells harboring pBF55-type plasmids were induced to overexpress various alleles of *Gpi8p* by exposure to  $\text{CuSO}_4$  at 37 °C for 1 h. Thereafter, aliquots of cells were taken for metabolic labeling with [ $^3\text{H}$ ]Ins for 60 min at 37 °C. The relative quantities of CP1 and CP2 were determined by radioscanning and are given as a percentage of the total radioactivity in the whole lipid extracts. The amounts of *Gpi8p* at the beginning of the labeling were obtained by densitometric analysis of the 46–50 kD region in the Western blots shown in Figure 2a and are expressed as fold increase over the normal, physiological level of *Gpi8p* in nontransfected cells. Panel b:  $\Delta\text{gpi8}$  cells were kept alive by pBF53-type plasmids containing wt or mutant alleles of *GPI8* under the control of the natural *GPI8* promoter. Cells grown at 24 °C were preincubated for 10 min at 37 °C and labeled with [ $^3\text{H}$ ]Ins during 1 h. Lipids were extracted, desalted, and separated by TLC. Radioactivity was visualized by fluorography and quantitated by radioscanning.

and C199A were expressed from a single copy vector under the control of the *CUP1* promoter or when C199A was integrated into the chromosomal *ura3-1* gene and expressed under the *GALI-10* promoter (not shown). These experiments indicate that overexpression of C199A and H157A rapidly interferes with GPI anchor attachment to proteins and that it is this cessation of GPI anchoring that arrests the growth of cells overexpressing C199A or H157A.

The accumulation of CP2 in S60A, S60C, and S60→C59 indicates that these mutants also have a small dominant negative effect on GPI anchor attachment. When the complementing alleles S60A, S60C, and even H54A were expressed either from a single or a multicopy vector under their natural promoter in  $\Delta\text{gpi8}$ , the cells showed accumulation of CP2 at 37 °C, but not at 24 °C (Figure 4b and data

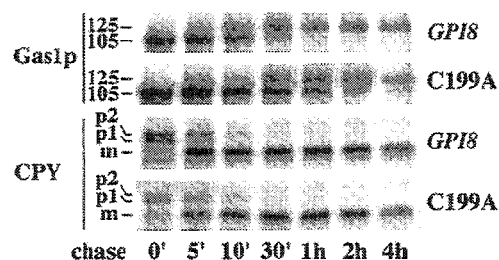


FIGURE 5: Maturation of Gas1p is delayed in wt cells overexpressing the C199A allele of *GPI8*. FBY577 and FBY578 cells harboring an integrated copy of wt *GPI8* or of C199A under the control of the *GALI-10* promoter, respectively, were grown to exponential phase at 24 °C in selective SDaa medium. Cells were washed twice with distilled water, resuspended in SGaa medium and cultured for 10 h at 37 °C in order to induce expression of recombinant Gpi8p. Then, the cells were pulse-labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 10 min and chased for the indicated times. Gas1p and CPY were immunoprecipitated from the lysates and were revealed by SDS-PAGE and fluorography.

not shown). Yet, all transformants grew well at 37 °C. Such cells thus resemble the original *gpi8-1* mutant which, at 37 °C, grows well, while accumulating CP2. The results confirm that these mutant alleles of Gpi8p are only partially functional.

We wanted to check if any perturbation caused by point mutations in Gpi8p is able to uncouple the proteolytic removal of the C-terminal GPI anchoring signal from the process of GPI attachment. For this reason, we tried to detect soluble GPI proteins in extracts or culture media of wt cells overexpressing S60→C59, S60A, S60C, or C199A. By Western blotting, we found that these cells indeed secrete small amounts of proteolytic fragments of Gas1p, Cwp1p, or Yap3p, but the same amounts of the same fragments are also secreted by control cells transfected with an empty vector or cells overexpressing wt Gpi8p (not shown).

We further tested a library of random mutagenized *gpi8* alleles by overexpressing them in wt cells and screening colonies for the secretion of soluble forms of an artificial GPI protein consisting of the guar  $\alpha$ -galactosidase and the C-terminal part of  $\alpha$ -agglutinin (kindly provided by M. P. Schreuder; (38)). Among 27 000 colonies tested, none secreted detectable amounts of  $\alpha$ -galactosidase (Urs Meyer and Markus Britschgi, unpublished).

**Accumulation of Immature Gas1p in the Presence of Mutated Gpi8 Proteins.** Impairment of GPI anchor attachment in *gpi* mutants can lead to the accumulation of immature GPI proteins such as the 105 kD form of Gas1p (10). The 105 kD Gas1p exits the ER and reaches the Golgi only after being attached to a GPI anchor. There, its N- and O-glycans are elongated, thus giving rise to the mature 125 kD form of Gas1p (27, 39, 40). When anchor attachment does not occur, immature Gas1p is retained in the ER and thus stays at 105 kD. The ratio of 105 kD/125 kD Gas1p in cell extracts can be probed by Western blotting with anti-Gas1p antibody. In wild-type cells, after strong induction of *gpi8* mutant alleles at 37 °C during 1 h, the 105 kD/125 kD ratios of Gas1p were slightly higher than in nontransfected wt cells, but a similar increase of the 105 kD/125 kD ratio was also seen in control cells in which wt Gpi8p was overexpressed (not shown). However, as shown in Figure 5, pulse-chase experiments clearly indicated a retardation of Gas1p maturation after induction of Cys199A. This effect

was specific for Gas1p, since the maturation of proCPY from p1 to p2 and to the mature form occurred with normal kinetics indicating normal transport rates for proCPY from ER to Golgi and to the vacuole (41). Thus, it seems that a problem in GPI anchoring is more readily observed by pulse-chase experiments than by measuring the 105 kD/125 kD ratio of Gas1p.

To test the partially functional alleles, we examined the 105 kD/125 kD ratio of Gas1p in  $\Delta$ *gpi8* expressing S60C or S60A at physiological levels and found it to be the same as in nontransfected W303 wt cells, even if cells were grown at 37 °C (not shown). Also, whereas overexpression of wt Gpi8p lowered the abnormally high 105 kD/125 kD ratio of Gas1p in *gpi7-gpi8* at 30 or 37 °C, all of the partially functional mutant alleles failed to do so but they did not increase this ratio either (not shown).

**Phenotypic Correlates of Various Degrees of Gpi8p Depletion.** The above results say that different mutant alleles, if overexpressed in wt cells or if replacing wt Gpi8p, generate different phenotypes of variable severity. We wanted to ascertain that this was due to the variable severity of the functional deficit in Gpi8 activity rather than to qualitatively different deficits in the various alleles. To do so, we constructed a  $\Delta$ *gpi8* strain containing a chromosomally integrated copy of *GPI8* under the control of the *GALI-10* promoter and shifted cells growing on galactose to glucose containing medium. As can be seen in Figure 6a, Gpi8p disappears rapidly. When we quantitate the results and calculate the rate of degradation of Gpi8p after correction for dilution of Gpi8p by ongoing cell division, we find that Gpi8p is turned over with a half time of 2.2 h during the first 3 h, whereas it is degraded more slowly and with a constant half time of 9.3 h during the following 3 h periods. An even more drastic difference in degradation rates between Gpi8p expressed at high and normal levels was observed when overexpressing myc-tagged versions of either wt *GPI8* or C199A in wt cells: excess Gpi8p, irrespective of its functionality (wt or C199A), was rapidly degraded within 1 h, but this rapid degradation was followed by a very slow phase of degradation of residual molecules (not shown). The more rapid degradation of Gpi8p during the first 3 h of depletion in Figure 6b may be related to the shift from galactose to glucose or may reflect the fact that the overexpressed Gpi8p is located outside the hypothetical transamidase complex, whereas Gpi8p at physiological levels resides within this complex.

Side by side comparison of cell extracts shown in Figure 6 with wild-type cell extract allowed to determine that cells containing *GPI8* under the control of the *GALI-10* and growing on galactose overexpressed Gpi8p 11.4-fold and that wt levels of Gpi8p were reached 4.5 h after the shift to glucose. Further depletion of Gpi8p led to a gradual decrease of growth rate and of plating efficiency with some cells remaining viable for up to 13 days (Figure 6h, not shown). It is conceivable that the minimal transcription from the glucose repressed *GALI-10* promoter may produce enough Gpi8p to prevent immediate cell death. Since a majority of GPI proteins are cell wall proteins, we tried to compensate for the expected cell wall fragility of Gpi8p depleted cells by the addition of sorbitol to the medium. This, however, did not increase the survival of Gpi8p depleted cells (not shown).

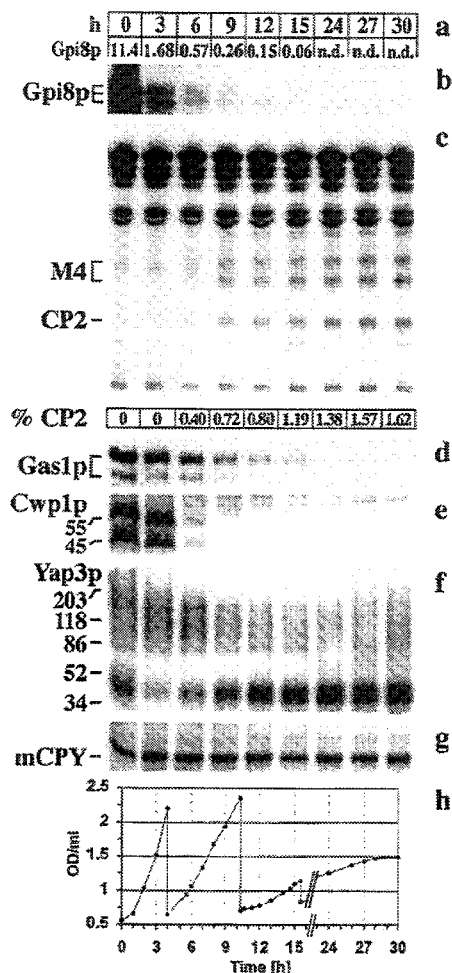


FIGURE 6: Time course of phenotypic changes during depletion of Gpi8p. FBY164 cells ( $\Delta gpi8/ura3-1::URA3-GAL1-10-GPI8$ ) were grown to exponential phase at 24 °C in SGaa medium. Expression of Gpi8p was subsequently blocked by shifting cells to glucose medium. Cultures were continued at 37 °C and periodically diluted with fresh medium to maintain the cell concentration in the range of 0.5–2.5 OD<sub>600</sub>/mL. Cell growth was followed by optical density measurements (OD<sub>600</sub>) and by cell counting. These two methods gave concordant results and indicated a constant ratio of 1.3–1.5 × 10<sup>7</sup> cells/OD<sub>600</sub> throughout the entire experiment (Panel h). Over a time period of 30 h following the shift to glucose, aliquots of cells were removed at 3 h intervals to monitor depletion of Gpi8p (Panel b) and the accumulation of immature forms of GPI proteins as well as of CPY by Western blotting and densitometry (Panels d–g). Numbers in Panel a define fold overexpression of Gpi8p as compared to W303-1B grown under the same conditions (n.d., not detectable). The detection limit for Gpi8p was at 6% of wt levels. The carbon source had no influence on the amount of Gpi8p in wt cells. To follow the accumulation of GPI precursor glycolipids, aliquots of cells were removed and labeled with [<sup>3</sup>H]Ins for 60 min (Panel c); the relative quantities of CP2 as determined by radioscanning are given as a percentage of the total radioactivity in the extracts.

CP2 accumulation became detectable in cells after 6 h on glucose, which, at the beginning and end of the 1 h labeling period, had 57 and 45% of the physiological amount of Gpi8p present in wt cells. CP2 accumulation continued to increase even after Gpi8p had fallen below the detection limit (24 h) (Figure 6c). This cannot be explained by an enhanced uptake of [<sup>3</sup>H]Ins due to a lower endogenous production of Ins at later time points, since the incorporation of [<sup>3</sup>H]Ins into lipids was the same at all time points (35% of the total radioactivity

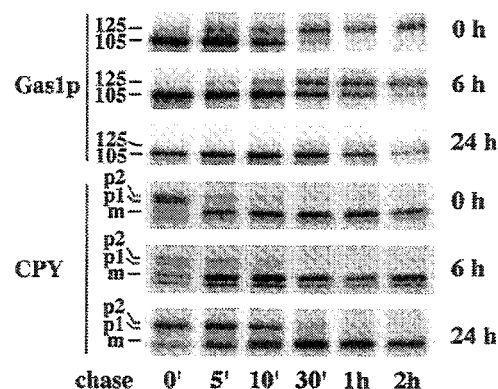


FIGURE 7: Depletion of Gpi8p blocks maturation of Gas1p. FBY164 cells were depleted of Gpi8p as described in Figure 6. Samples of the cell culture were harvested 0, 6, and 24 h after shifting to glucose. Maturation of Gas1p and CPY was analyzed at 37 °C by pulse chase labeling as described in Figure 5.

added to cells). The two most likely interpretations are that Gpi8p is continuously decreasing between 15 and 30 h so that less and less of CP2 can be used for GPI anchoring or else, that the GPI anchoring deficiency somehow leads to an upregulation of CP2 biosynthesis. By Western blotting, Gas1p was found to be depleted together with Gpi8p with no sign of accumulation of the immature 105 kD form (Figure 6d). Rapid disappearance was also observed for core glycosylated forms of Cwp1p, a GPI-anchored cell wall protein, which is released as a 55–60 kD protein from the cell wall (42, 43) and accumulates as a 45 kD form in *sec18* cells at 37 °C (Figure 6e). Another GPI protein of the plasma membrane, Yap3p was found to accumulate as a heterogeneous band of about 34 kD, which is not usually seen in wt cells (Figure 6f). However, *sec18* cells rapidly accumulate large amounts of a 34 kD form of Yap3p upon shift to 37 °C (not shown), although the open reading frame of *YAP3* predicts a 60 kD translation product and the endoglycosidase H-treated mature protein has a molecular weight of 68 kD (44). Thus, the 34 kD form may represent a proteolytic fragment arising when Yap3p is retained in the ER. The vacuolar protein CPY was not influenced by the depletion of Gpi8p. We also correlated the Gpi8p depletion with the maturation kinetics of Gas1p. As shown in Figure 7, after 6 h of Gpi8p depletion, when the amount of Gpi8p was around 57% of wt, we already observed a very significant delay in Gas1p maturation, and after 24 h of depletion Gas1p seemed to get degraded in its immature 105 kD form, whereas CPY was still matured, albeit with about 3-fold slower kinetics than normally.

## DISCUSSION

The GPI transamidase has not yet been purified in an active form and mutant screens have so far only identified two genes, *GAA1* and *GPI8*, which accumulate complete precursors and unprocessed proforms of GPI proteins. The list of potential functions of *GAA1* and *GPI8* comprises (i) translocation of CPs from the cytosolic to the luminal surface of the ER, (ii) recognition of the protein or lipid substrate, (iii) proteolytic removal of the anchor attachment signal peptide, or (iv) attachment of the GPI (9). The 25–28% sequence identity between *yGPI8* and the original class 13 Cys protease family has suggested that *yGPI8* may be

involved in the proteolytic removal of the anchor attachment signal peptide (28), especially since one member of the C13 protease family, the jack bean asparaginyl endopeptidase, shows transamidase activity *in vitro* and is believed to be involved in a transamidation, i.e., a transpeptidation reaction *in vivo* (45).

The data in this report indicate that Cys199 of *GPI8* is located in a context that is homologous to the active site Cys of caspases and is a functionally highly important amino acid. Indeed, mutation of C199 to Ala completely abolishes the activity of *GPI8*. This drastic effect of the replacement of a single sulfhydryl by a hydrogen suggests that C199 is either the active site or that it is forming a strategically important intra- or interchain disulfide bridge. An intrachain bridge is highly unlikely, since Gpi8p has no other Cys that could act as partner for the formation of a disulfide bridge; mutation of the only other conserved Cys of the *GPI8* subfamily, Cys85, does not abolish *GPI8* function (Table 1). Moreover, as judged from the kinetics of turnover, the stability of the C199A and the *GPI8* wt alleles are the same (not shown). Finally, in another context, we have deleted Cys373 and found that it is not required for catalytic activity (P. Fraering, in preparation). Cys199 also does not form an interchain disulfide bridge with another protein, since Gpi8p has the same mobility in SDS-PAGE, whether reducing agent is present or not. Cys199 most likely is the active site of Gpi8p also because in a recent report, the mutation of the corresponding Cys in another C13 protease, the murine lysosomal legumain, similarly induces a complete loss of activity (46).

Mutation of His157 also completely abolishes the functionality of Gpi8p, whereas mutation of the only other almost entirely conserved His of the C13 family, His54, only leads to partial loss of Gpi8p function. It, therefore, is likely that deprotonation of Cys199 is mediated by His157 and not by His54. A further strong argument in favor of His157 derives from the fact that it is the only His that is followed by Gly and that this feature is conserved in the whole C13 family. Indeed, the analogy with caspases suggests that Gly158 also is of strategic importance, since all caspases contain a Gly following the active site His to create the so-called oxyanion hole, which transiently accommodates the negatively charged carbonyl anion generated by the nucleophilic attack of the active site Cys on the carbonyl group of the P1 residue (22). (In other protease families, the glycine involved in the formation of the oxyanion hole is not vicinal to the active site His (23, 47)). These findings suggest that, contrary to an earlier prediction (16), the Cys and His residues corresponding to C199 and H157 of  $\gamma$ *GPI8* represent the active site residues in all members of the C13 protease family; a possible exception remains *S. mansoni* hemoglobinase B, which lacks the corresponding Cys residue. However, it is unclear if this protein contains any proteolytic activity (13, 48).

The proposed proteolytic activity of Gpi8p can be assumed to be of relatively broad substrate specificity, since the comparison of primary sequences at and around the cleavage site ( $\omega$ -site) of natural GPI proteins as well as the systematic alteration of this region have shown that small amino acids are required or at least preferred at  $\omega$ ,  $\omega+1$ , and  $\omega+2$  sites, but that in other positions around the  $\omega$  site about any amino acid can be tolerated (40, 49–52). We, therefore, may

anticipate the existence of some mechanism to prevent the cleavage of unrelated proteins that are anchored by classical transmembrane domains. This mechanism may depend on additional subunits regulating the activity of Gpi8p.

Out of our three completely nonfunctional alleles of *GPI8*, C199A, H157A, and S60→C59, the former two have a strong, dominant negative effect on the growth rate. Since concomitant overexpression of wt Gpi8p reverses this inhibition, it would appear that growth inhibition is not due to the presence of nonfunctional Gpi8 proteins *per se* but rather to the relative preponderance of functional over nonfunctional Gpi8 proteins. These data can best be explained by assuming that Gpi8p functions as part of a heteropolymeric transamidase complex, containing several subunits, the implication being that the copy number of some of these subunits cannot be upregulated and that the absolute number of transamidase complexes per cell remains the same even when functionally impaired alleles of *GPI8* are overexpressed. Alternatively, we may assume that Gpi8p, to be functional, has to form a homopolymeric complex in which all individual subunits would have to be fully functional. The existence of a heteropolymeric complex would also explain why the overexpression of various partially functional alleles cannot restore normal growth at 37 °C to the *gpi7-gpi8* double mutant and cannot lower its abnormally high 105 kD/125 kD ratio of Gas1p and why these alleles, when overexpressed in  $\Delta$ *gpi8*, cannot prevent the accumulation of CP2 (data not shown). In all these cases, the hypothetical transamidase complexes would be functionally impaired because they contain mutated Gpi8p's and the cell's overall transamidase activity would be reduced because the number of complexes is limited by the unchanging number of other subunits.

Thus, to our mind, the strong dominant negative effect of certain *GPI8* alleles on cell growth and the correlated accumulation of CP2 strongly suggest the existence of a transamidase complex, but the existence of this complex needs to be confirmed by biochemical evidence.

One obvious candidate for a further transamidase subunit is Gaa1p (9). Gpi8p and Gaa1p both seem to be intimately involved in the attachment of GPI anchors to proteins, since mutations in these genes produce a similar phenotype and are synthetically lethal (10). Overexpression of Gaa1p has been shown to improve the efficiency of anchor addition to Gas1p<sup>N506C</sup>, a Gas1p mutant in which the natural anchor acceptor site had been mutated from Asn to Cys (9). This raises the possibility that Gaa1p is involved in the recognition of the protein substrate (9). If the transamidase complex were a heterodimer made of Gaa1p and Gpi8p, then we would expect that the dominant negative effect of C199A overexpression can be counteracted by simultaneous overexpression of Gaa1p. We, however, found that the growth inhibition caused by overexpression of C199A in wt cells was not relieved by simultaneous overexpression of Gaa1p using strains and plasmids generously provided by Dr. Howard Riezman, although Gaa1p overexpression from the same plasmid slightly but significantly improved the GPI anchoring of a Gas1p<sup>N506C</sup> mutant allele and, in other experiments, complemented the thermosensitive growth phenotype of *gaa1* (not shown). Also, overexpression of wt Gpi8p did not reduce the accumulation of the immature 105 kD form of Gas1p<sup>N506C</sup>, whereas simultaneous overexpression of Gpi8p and Gaa1p

did reduce the accumulation of the immature 105 kD form of Gas1p<sup>N506C</sup>, but no more than the overexpression of Gaa1p alone (not shown). These negative data suggest that other subunits beyond Gpi8p and Gaa1p may equally be required to form functional transamidase complexes.

The dominant negative effect of C199A or H157A overexpression in wt cells rapidly leads to an accumulation of CP2 (Figure 4a) and a delay in the maturation of newly synthesized Gas1p (Figure 5). Over time, it also leads to the arrest or severe reduction of cell growth (Figure 3). The same order of events is also observed when Gpi8p is depleted (Figure 6). A priori, it is quite conceivable that the number of functional transamidase complexes could be significantly reduced, causing the accumulation of CP2 and of immature GPI proteins, but that the increased concentration of these substrates would make up for the reduced number of functional transamidase complexes and that, nevertheless, a normal amount of GPI proteins are anchored per unit of time. This is clearly not the case, neither when we overexpress C199A or H157A nor when we deplete Gpi8p, precisely because both of these conditions lead to a reduction of the growth rate already if Gpi8p is depleted by 50%, because they lead to a complete growth arrest after 48–72 h and because GPI proteins such as Gas1p and Cwp1p even disappear quite rapidly (Figure 6d and 6e). Thus, it seems reasonable to assume that the concomitant CP2 accumulation and delay of Gas1p maturation reflects in all cases a decrease in the rate of GPI transfer onto proteins.

Mutant alleles S60C and S60A rescue  $\Delta gpi8$  but are selected against in *gpi7–gpi8* (Figure 2b) and their overexpression in wt cells causes CP2 accumulation (Figure 4a). This dominant negative effect is particularly pronounced and intriguing for S60A, a mutation that only removes a single oxygen, since Ser60 lies in a region that, even in the *GPI8* subfamily, is only moderately conserved and since *C. elegans GPI8* has an Ala at the position corresponding to Ser60. It seems that the function of Ser60 can partly be accomplished by a Cys, since the defect of S60C is less severe than the one of S60A. If we interpret the data assuming the existence of a transamidase complex, then we are led to believe that S60 is important for the catalytic activity of yGpi8p rather than for its insertion into the transamidase complex. (Mutations preventing the integration of Gpi8p into a transamidase complex would not be expected to exhibit a dominant negative phenotype). The S60→C59 allele on the other hand, although completely unable to rescue  $\Delta gpi8$ , seems to have very little dominant negative effect, suggesting that this allele may be improperly folded or may be unable to enter the transamidase complex.

It is interesting to consider the kinetics of CP2 appearance upon induction of *CUP1* promoter driven C199A. CP2 accumulation is quite obvious already after 1 h of induction (Figure 4a) and the degree of accumulation at this stage (1.11%) corresponds, when compared to the CP2 accumulation during the Gpi8p depletion experiment, to a reduction of Gpi8p down to about 10% of normal (Figure 6c). Since the half-life of normal Gpi8p has been estimated to be in the order of 9 h, this rapid decrease of transamidase activity cannot be explained simply by replacement and dilution of transamidase complexes containing wt Gpi8p by new ones containing C199A. The result rather suggests that newly made C199A can get incorporated into preexisting complexes

implying that these complexes are dynamic in nature.

The analysis of the phenomena occurring during Gpi8p depletion puts the phenotypes obtained with the various *gpi8* alleles into perspective. The biphasic degradation rate may reflect the degradation of Gpi8p molecules excluded from and integrated into the transamidase complex, respectively, but further studies will have to address this point. At any event, it would appear that the physiological levels of Gpi8p are not far above the threshold at which transamidase capacity becomes limiting because already a 50% reduction of physiological Gpi8p levels leads to CP2 accumulation and a delay of Gas1p maturation. On the other hand, it seems that cell growth can continue for a few generations in the presence of very low transamidase activity, probably because several essential GPI proteins are normally present in large excess.

The use of dominant negative alleles may allow to construct transgenic animals with conditional or tissue specific deficiencies of GPI anchoring in order to further understand the role of GPI anchoring (53, 54). Our own studies are currently directed at the identification of other subunits of the transamidase complex.

## ACKNOWLEDGMENT

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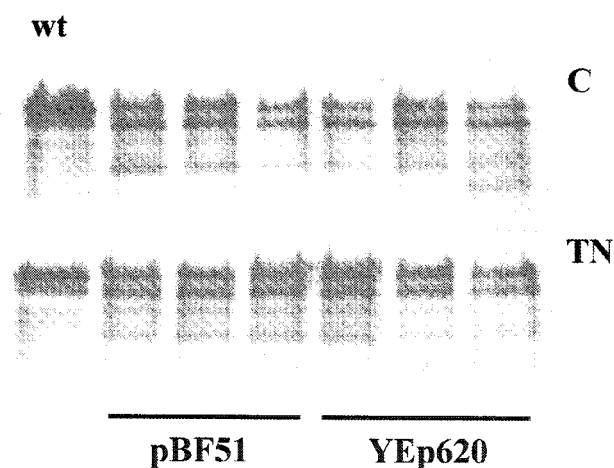
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### 3.1.2. Additional results performed with Gpi8p or in the context of the active site determination

#### 3.1.2.1. Overexpression of Gpi8p does not suppress the partial GPI anchor attachment defect of *gas1* GPI signal sequence mutants

Hamburger *et al.* (1995) have developed a series of Gas1p mutants having an altered GPI signal sequence which are not processed efficiently by the transamidase and showed that overexpression of Gaa1p leads to a partial suppression of the GPI anchoring defect. By using the same strains and plasmids, we wanted to test whether overexpression of Gpi8p also improved anchor addition to these Gas1p mutants. Therefore, the multicopy plasmid pBF51 overexpressing *GPI8* under the control of its natural promoter was transformed into  $\Delta gas1$  cells (RH 392-3A) either containing plasmid pCNYCG-C expressing the  $\omega$  site mutant N506C or plasmid pCNYCG-TN containing a spacer region mutation in which amino acids T and N were deleted. Cells were incubated and proteins extracted as described (Hamburger *et al.*, 1995). Immunoblotting with anti-Gas1p antibodies and quantification of the immature 105 kD immature and the 125 kD mature form of Gas1p protein revealed that overexpression of Gpi8p could not significantly suppress the GPI anchoring defect on these mutants. This may be an indication that Gaa1p rather than Gpi8p is involved in specific recognition of the GPI-SS (Figure 1).



**Figure 1. Overexpression of Gpi8p does not suppress the partial GPI anchor attachment defect of Gas1p mutants with an altered GPI-SS.**

The relative amount of the upper 125 kD mature band was determined. For wt Gas1p a number of 80 % was obtained, whereas for all transformants expressing the  $\omega$  site mutant N506C and for all transformants expressing the TN mutant the relative amount of mature Gas1p was in the range of 46 and 55%, respectively, whether Gpi8p was overexpressed

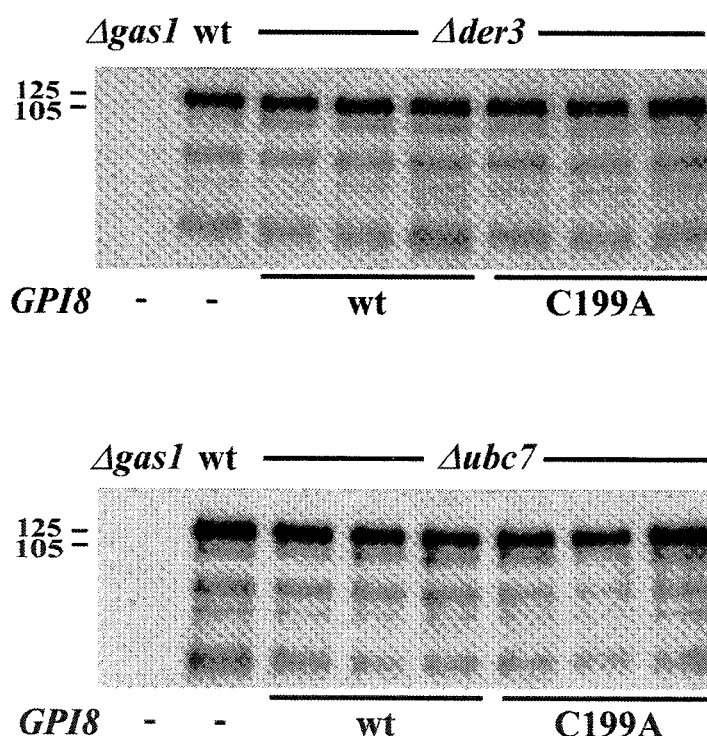
(pBF51) or not (YEp620). Overexpression of Gpi8p was checked by immunoblotting with anti-Gpi8p (data not shown).

Furthermore, co-overexpression of Gpi8p and Gaa1p did not increase the suppressive effect of Gaa1p overexpression on the GPI anchoring defect of these GPI-SS mutants (data not shown).



### 3.1.2.2. Unprocessed immature Gas1p is not degraded by proteasomes

It has been shown that overexpression of the dominant-negative alleles of *GPI8*, C199A and H157A, leads to growth arrest and accumulation of CP2. Furthermore, it was shown by pulse-chase experiments that cells overexpressing the C199A mutant or cells which were depleted of functional Gpi8p accumulate the 105 kD immature ER form of the GPI model protein Gas1p. However, immunoblotting of whole cell protein extracts from the same strains with anti-Gas1p antibodies did not show significant accumulation of this 105 kD band, suggesting that immature Gas1p may be rapidly degraded (Meyer *et al.*, 2000). Since GPI anchor attachment occurs in the endoplasmatic reticulum, we wanted to know whether components of the ER degradation machinery are involved in scavenging immature Gas1p. CPY\*, a mutant form of the vacuolar carboxypeptidase Y, was shown to accumulate in the ER when the function of *DER3* was impaired (Knop *et al.*, 1996; Bordallo and Wolf, 1999). *DER3* and *DER1* are genes which mediate the retrograde transport of proteins, which are not correctly folded in the ER, through the translocation pore out of the ER towards the proteasome degradation pathway (Plempner *et al.*, 1999; for review Plempner and Wolf, 1999). To analyze whether Der3p or Ubc7p, a ubiquitin-conjugating enzyme (E2) (Cook *et al.*, 1997), are involved in degradation of immature Gas1p, we overexpressed the dominant-negative C199A mutant in  $\Delta der3$  (FBY581) and  $\Delta ubc7$  cells (FBY582).



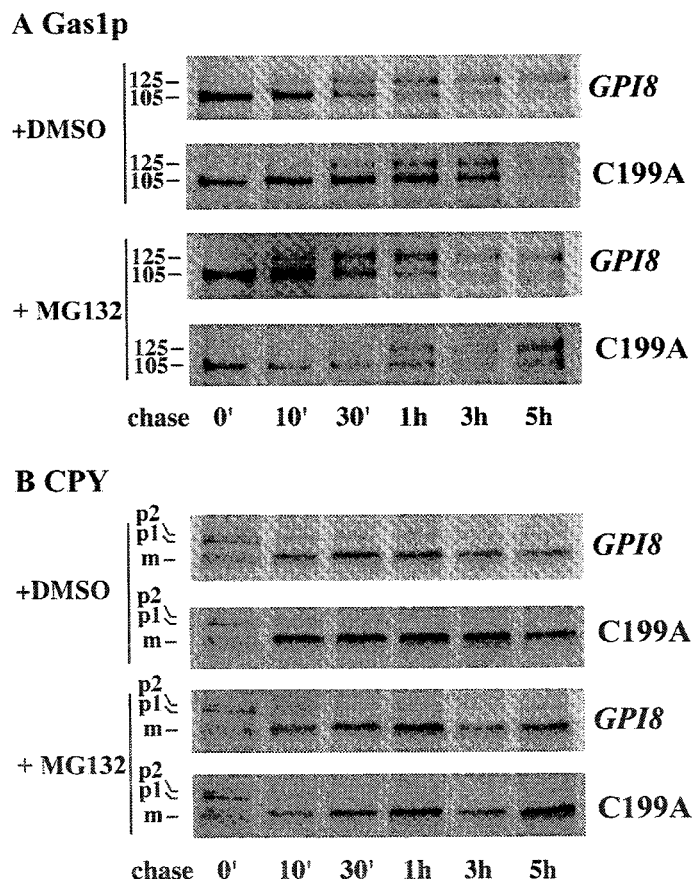
**Figure 1. Overexpression on the dominant-negative C199A mutant in  $\Delta der3$  and  $\Delta ubc7$  did not lead to an increased accumulation of Gas1p.**

Deletant strains expressing *GPI8* alleles either from plasmid pBF54 (wt) or pBF54C199A (C199A) and W303-1B wt cells which were grown to exponential phase in selective SDaa medium were harvested and resuspended in fresh selective SDaa medium containing 500  $\mu$ M  $\text{Cu}^{2+}$  to induce overexpression of *GPI8* alleles. After a preincubation of 1 h at 24 °C, the

incubation was prolonged for 6h at 37 °C and stopped by the addition of NaF/NaN<sub>3</sub> (10 mM each). Whole cell protein extracts together with a whole cell extract of  $\Delta gas1$  cells (RH 392-3A) grown to exponential phase at 24 °C were separated by SDS-PAGE. Gas1p was detected by immunoblotting with anti-Gas1p antibodies.

As it can be seen in Figure 1, overexpression of C199A in the genetic background of  $\Delta der3$  and  $\Delta ubc7$  did not lead to an increased accumulation of the immature 105 kD band of Gas1p. This suggests that both Der1p and Ubc7p protein are not directly involved in degradation of unprocessed Gas1p.

In addition, we analyzed whether the 105 kD band of Gas1p, which is accumulated in cells overexpressing the C199A, is stabilized by the well-described proteasome inhibitor MG-132. It has been shown that  $\Delta erg6$  are especially permeable to several proteasome inhibitors including MG-132 (Lee and Goldberg, 1998). Erg6p is the putative *S*-adenosylmethionine: $\Delta^{24}$ -methyltransferase of the ergosterol biosynthesis pathway and catalyzes C-24 methylation. Moreover, it is required for normal membrane function (Gaber *et al.*, 1989). We overexpressed the C199A mutant in an  $\Delta erg6$  background and followed Gas1p labeled with [<sup>35</sup>S]-methionine/[<sup>35</sup>S]-cysteine in the presence and absence of MG-132 by pulse-chase analysis.



**Figure 2. Maturation of Gas1p is delayed in wt cells overexpressing the C199A allele of GPI8, but is not affected by MG-132.**

$\Delta erg6$  (FBY580) harboring pBF54-type single copy vectors expressing either wt GPI8 or C199A under the control of the *CUP1* promoter were grown to exponential phase at 24 °C in

selective SDaa medium. Cells were then harvested and preincubated in selective SDaa medium containing 500  $\mu\text{M}$  of  $\text{Cu}^{2+}$  for 2h at 37°C to induce overexpression of *GPI8* alleles. Cells were harvested and incubated in labeling medium containing 250  $\mu\text{M}$  of  $\text{Cu}^{2+}$  for 20'. Then, the cells were pulse-labeled for 10' with [ $^{35}\text{S}$ ]methionine and [ $^{35}\text{S}$ ]cysteine in the presence of MG-132 or DMSO (control) and chased for the indicated times. Gas1p and CPY were immunoprecipitated from the lysates and were revealed by SDS-PAGE and fluorography (for protocols (Meyer *et al.*, 2000)).

As it can be seen in Figure 2, MG-132 did not significantly increase the accumulation of the immature 105 kD band of Gas1p; this was the case in both  $\Delta\text{erg6}$  which overexpress wt *GPI8* and in  $\Delta\text{erg6}$  cells which overexpress the mutant allele C199A. This strongly suggests that the partial degradation of the immature 105 kD band of Gas1p accumulated in cells overexpressing C199A or in cells depleted of Gas1p is not dependent on proteasomes. However, it has to be mentioned that the maturation of Gas1p in the analyzed  $\Delta\text{erg6}$  strain was slower than in W303-1B cells overexpressing the wt *GPI8* allele (Meyer *et al.*, 2000). The generation time of  $\Delta\text{erg6}$  was longer than that of W303-1B.

## 3.2. Screening for proteolytically active *gpi8* mutants

### 3.2.1. Introduction

The previous chapter described the experimental identification of the amino acids C199 and H157 as active site residues of Gpi8p based on sequence comparison with the known cyteine protease families C13, consisting mainly of plant cysteine proteases, and C14, being the caspase family of cysteine aspartic acid-specific proteases which had been extensively described to be involved in apoptosis (classification of peptidase families and index of peptidase entries in SWISS-PROT; (Rawlings and Barret, 1994)). This finding supports the hypothesis that Gpi8p might act in a reaction similar to that of proteases, and together with the phenotype of impaired GPI anchor attachment of *gpi8* mutants or wt cells oveexpressing mutant Gpi8p, suggests that Gpi8p is directly involved in the proteolytic removal of the GPI anchoring signal (Meyer *et al.*, 2000).

In preliminary experiments with purified Gpi8p, no proteolytic activity towards small commercial proteinase substrates was detected, even not when millimolar concentrations of hydrazine or free ethanolamine were added as a nucleophile (Benghezal *et al.*, 1996). Further experiments with more physiological substrates, i.e. mimicking the sequence around the  $\omega$  site of yeast GPI proteins, or peptides, which are cleaved by a large range of peptidases, in the presence or absence of putative nucleophilic agents such as hydrazine, hydroxylamine, ethanolamine and reducing agents to preserve the active site cysteine did not reveal any proteolytic activity of Gpi8p *in vitro* (Fraering *et al.*, submitted). This might have several reasons: First, it is likely that the enzyme-bound substrate is shielded from the aqueous environment in order to prevent hydrolysis of the thioester bond (carbonyl intermediate) between the enzyme and the substrate, and that this thioester bond can only be attacked by a nucleophile specifically bound to the enzyme (Meyer *et al.*, 2000). This postulate stems from the fact that no immature GPI proteins with a cleaved GPI-SS but lacking a GPI anchor are observed in living systems. However, in a microsomal *in vitro* translation-translocation GPI addition system it was shown that prominiPLAP gets proteolytically processed at its C terminal end without the addition of a GPI, presumably due to catalytic action of the transamidase. (Maxwell *et al.*, 1995b). Second, no proteolytic activity of purified Gpi8p was found, possibly because additional components are required to activate Gpi8p. These may consist of additional subunits of the putative GPI:protein transamidase complex such as Gaa1p, or other co-factors. Third, the negative result might have been due to the fact that Gpi8p lost its correct conformation and therefore, its activity, during the purification procedure. Fourth, Gpi8p may not display proteolytic activity under any conditions, and hydrolysis of prominiPLAP reported by Maxwell *et al.* (1995) was independent on Gpi8p.

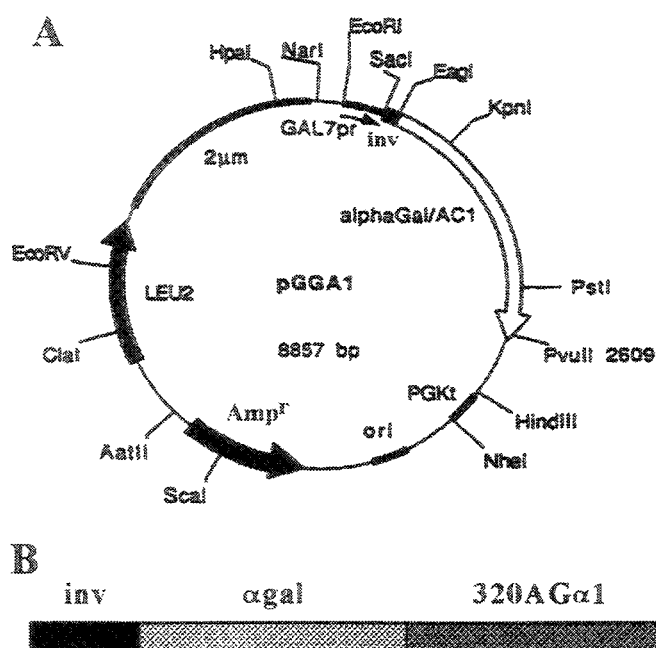
We estimated the fourth possibility as to be the most unlikely, and decided by avoiding the first three possibilities to perform a screen which was designed to select for *gpi8* mutants showing an *in vivo* proteolytic activity. Such a mutant would produce soluble C terminally cleaved GPI proteins without GPI modification by uncoupling the formation of a carbonyl intermediate from the transamidation onto a preformed GPI precursor. For such a screen, wt cells expressing a synthetic GPI reporter protein consisting of  $\alpha$ -galactosidase fused to the C terminal part of  $\alpha$ -

agglutinin were transformed with a library of random mutagenized *gpi8* alleles and selected for secretion of the GPI reporter protein.

### 3.2.2. Results

#### *The GPI reporter protein*

A previously published fusion protein of the N terminal signal of yeast invertase attached to the reporter protein guar  $\alpha$ -galactosidase and the 320 C terminal amino acids of the yeast cell wall protein  $\alpha$ -agglutinin was expressed under the control of the galactose-inducible promoter *GAL7* (Figure 1). Stable expression of the GPI reporter protein and integration in the cell wall of yeast wt cells was monitored on selective SGalaa plates containing the dye X- $\alpha$ -Gal (5-bromo-4-chloro-3-indolyl- $\alpha$ -D-galactose) in a concentration of 50  $\mu$ g/ml. Upon cleavage by  $\alpha$ -galactosidase, the dye becomes dark blue and - since the GPI reporter protein is covalently integrated into the cell wall - colorates the yeast colonies (Lu *et al.*, 1995b; Schreuder *et al.*, 1993).



**Figure 1. Plasmid pGGA1 expressing the GPI reporter protein.**

Plasmid pGGA1 (A) kindly provided by M.P. Schreuder expresses under the control of the *GAL7* promoter the fusion protein (B) consisting of an N terminal signal sequence for entry into the ER taken from the yeast periplasmic protein invertase, fused with the reporter protein guar  $\alpha$ -galactosidase and the 320 carboxyterminal amino acids of the yeast cell wall protein  $\alpha$ -agglutinin ( $\alpha$ -gal/AG $\alpha$ 1), the last containing the GPI-SS for GPI anchor attachment.

In contrast, colonies of wt cells transformed with the control plasmid pSY1 expressing the very same fusion protein but lacking the C terminal amino acids of  $\alpha$ -agglutinin remain white or become only weakly bluish, but show a blue halo which indicates secretion of the fusion protein lacking the GPI-SS (Schreuder *et al.*, 1993).

#### *Construction of the library with random mutagenized *gpi8* alleles.*

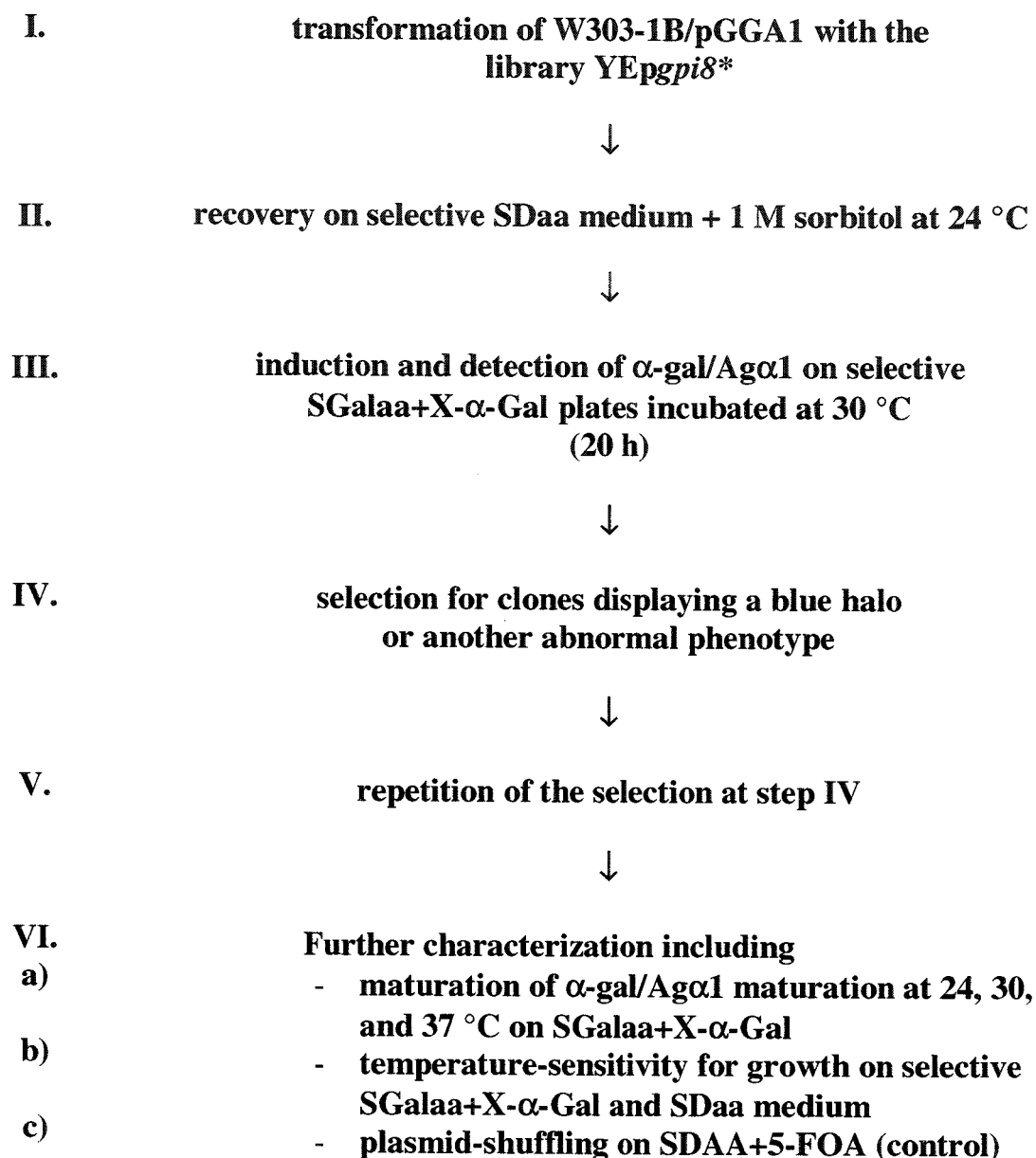
In order to guarantee proper insertion into the ER and increase the probability of correct subcellular localization and of precise integration into the putative GPI:protein transamidase complex, we mutated only the ER luminal part of Gpi8p (amino acid positions 26 to 385), but left the N terminal signal sequence and the C terminal part containing the transmembrane domain and a short cytosolic stretch of about 15 amino

acids intact (Figure 10, chapter 1.4.4.4.). Mutations were introduced the yeast *GPI8* gene by random PCR according to Leung *et al.* (1989), who used a modified PCR technique to mutagenize a defined DNA fragment of 250 to 1200 bp in length. In this report DNA amplification was performed under conditions which modified activity and fidelity of the *Thermus aquaticus* (Taq) DNA polymerase. Addition of extra MgCl<sub>2</sub>, of MnCl<sub>2</sub>, β-mercaptoethanol and unequal concentrations of dNTPs to the polymerase buffer resulted in a frequency of point mutations of approximately 2 %; the probability for transitions and transversions of base substitutions was well-balanced occurring with an approximate frequency of 50% each. The quality and the success of a PCR was always limited by the size of the synthetic DNA fragment; for a target region of more than 300 bp, the yield of mutants with single- and multiple-point mutations was higher than 90 % (Leung *et al.*, 1989).

By following this protocol, we mutagenized a fragment of 1283 bp being slightly longer than the fragments analyzed by Leung *et al.* (1989) and obtained a frequency of point mutations of approximately 0.7 % which was lower than expected but still made 7 to 8 mutations into the amplified DNA fragment of 1074 bp which was cloned into the YEp*GPI8* plasmid (Benghezal *et al.*, 1996) to generate the library containing mutagenized *gpi8* alleles. The approximate variability of the library was determined by counting the bacterial colonies growing after electroporation with the ligation products and plating of an aliquot on apart. This predicted more than 260'000 different *gpi8* alleles to be present in the library. The library contained only plasmids of the same size, no abnormal ligation products were found.

#### ***Selection procedure for proteolytic gpi8 alleles***

W303-1B wild type cells containing the GPI reporter protein expressing pGGA1 were then subjected to electroporation with the YEp*gpi8*\* library which constitutively overexpresses the *gpi8* alleles under the control of the wt *GPI8* promoter. The transformants were incubated after electroporation at 24 °C on selective SDaa medium containing 1 M sorbitol and were then subjected to a selection protocol (for summary see Figure 2).



**Figure 2. Selection procedure for proteolytic or dominant-negative *gpi8* mutants.**

W303-1B containing plasmid pGGA1 were transformed with the *gpi8* allele library (YEpgpi8\*, step I), plated onto selective SDaa medium + 1M sorbitol and incubated at 24 °C (step II). Medium sized colonies were then replica-plated onto selective SGalaa medium containing X-α-Gal (SGalaa+X-α-Gal) to induce and detect the expression of the GPI reporter protein (α-gal/AGα1; step III). Colonies displaying a blue halo or an abnormal phenotype (weak or no coloration of the colony, reduced growth) were selected (step IV) and in order to obtain reproducible results, subjected to another round of the same selection procedure (step V). Clones which did show twice an abnormal phenotype (steps IV and V) were further characterized (step VI) including analysis of α-gal/Agα1 maturation at different temperatures (24, 30, 37 °C and over longer time periods), growth phenotype on glucose medium (dependent only on the expression of the *gpi8* allele but not on that of α-gal/Agα1), and whether a normal coloration phenotype could be restored by shuffling out the *URA3*-based plasmid YEpgpi8\*.

More than 27'000 transformants (W303-1B/pGGA1/YEp*gpi8*\*; steps II/III) were screened for an abnormal maturation of the  $\alpha$ -galactosidase/ $\alpha$ -agglutinin fusion protein ( $\alpha$ -gal/Ag $\alpha$ 1) at 30 °C on SGalaa+X- $\alpha$ -Gal medium after an incubation of 20 h; incubation of transformants at 30 °C was prolonged up to 9 days in order to find additional clones abnormal  $\alpha$ -gal/Ag $\alpha$ 1 maturation phenotypes including halo formation. The first selection procedure (step IV) resulted in the isolation of approximately 800 candidates for an abnormal  $\alpha$ -gal/Ag $\alpha$ 1 maturation or abnormal growth on SGalaa+X- $\alpha$ -Gal medium at 30 °C. None of them showed halo formation similar to that observed for the positive control (W303-1B/pSY1/YEp*GPI8*). There was also no clone which showed at least a weak bluish halo different from the very weak halo detected in the negative control (W303-1B/pGGA1/YEp*GPI8*) after 1 to 2 days of incubation on SGalaa+X- $\alpha$ -Gal. This indicates that we could not identify a clone which secreted  $\alpha$ -gal/Ag $\alpha$ 1 in a significant manner among the 27'000 tested colonies.

Nevertheless, we decided to continue the analysis of the 800 abnormal clones. A lot of them seemed to be false positives due to differences in size of the colonies; small-sized colonies generally showed weak coloration and big colonies showed coloration which was so strong that halo formation was suspected. Furthermore, although most of the clones grew well at 24 °C on glucose medium, certain colonies displayed reduced or no growth at 30 °C on SGalaa+X- $\alpha$ -Gal medium which was speculated to be due to the temperature effect together with *gpi8* allele expression, the carbon source substitution or eventually also the expression of  $\alpha$ -gal/Ag $\alpha$ 1 together with the other, before mentioned stress factors.

To discard false positive clones, the same selection procedure was repeated (step V) by picking all 800 single colonies onto a plate with SDaa medium, incubating for 1 to 2 days at 24 °C in order to obtain well-growing, medium-sized colonies and replica-plating onto SGalaa+X- $\alpha$ -Gal medium to detect maturation of  $\alpha$ -gal/Ag $\alpha$ 1. The few clones which did not grow well on SDaa medium were suspected to be possible candidates for dominant-negative *gpi8* alleles and collected together with all clones which reproducibly showed abnormal maturation of  $\alpha$ -gal/Ag $\alpha$ 1, whereas all clones showing wt maturation of  $\alpha$ -gal/Ag $\alpha$ 1 were discarded. Altogether, 24 clones were found to display reproducibly an abnormal phenotype.

These 24 clones were then subjected to a final test for  $\alpha$ -gal/Ag $\alpha$ 1 maturation and growth on SDaa and SGalaa+X- $\alpha$ -Gal medium for prolonged period (step VI). In addition, we speculated that certain of our clones became white because they lost the pGGA1 plasmid from which the GPI reporter protein is expressed; this could also explain why certain clones did grow very slowly. Clones without pGGA1 would no more be able to synthesize leucine and could eventually survive by cross-feeding, at least during our tests. Loss of the plasmid YEp*gpi8*\* seemed more difficult to detect since these clones would show wt maturation of  $\alpha$ -gal/Ag $\alpha$ 1 (which can be detected even when cells grow slowly). However, we cannot exclude that we lost eventually some clones which expressed strong dominant-negative Gpi8p by counterselection during the selection procedure, although all tests – if possible – were done starting from original master plates and in a way that time for counterselection was minimal. To detect and discard clones which lost the pGGA1 plasmid, in all 24 selected clones, the YEp*gpi8*\* plasmid containing the *URA3* marker gene was forced out by plasmid shuffling on selective SDaa+5-FOA medium, and the clones were again subjected to analysis of  $\alpha$ -gal/Ag $\alpha$ 1 maturation on SGalaa+X- $\alpha$ -Gal medium. 6 out of the 24 clones did not grow at all on SDaa+5-FOA medium as was tested twice (clones 8, 9,



14, 15, 19 and 23; Table 1); for these clones it was not possible to prove that an abnormal phenotype for growth or  $\alpha$ -gal/Ag $\alpha$ 1 maturation was due to the expression of a *gpi8\** allele. It is possible that they had lost the capacity to take up uracile or that they had undergone recombination between the mutant and wt *GPI8* so that wt *GPI8* resided on the plasmid whereas only mutant forms of GPI8 were present in the genomic *GPI8* locus.

**Table 1. Growth and maturation of  $\alpha$ -gal/Ag $\alpha$ 1 on SGalaa+X- $\alpha$ -Gal (summary of steps VI a) and b)).**

clone	SGalaa+X- $\alpha$ -Gal 24 °C		SGalaa+X- $\alpha$ -Gal 30 °C		SGalaa+X- $\alpha$ -Gal 37 °C	
	20 h	3 d	20 h	3 d	20 h	3 d
1	+/w	++/w	-/w	-/w	-/w	-/b
2	+/w	++/w	-/w	-/w	-/w	-/w
54	-/w	+/w	-/w	+/w	-/w	+/w
6	+/bw	++/bw	+/bw	+/bw	-/bw	+/bw
10	+/w	++/bw	+/bw	++/bw	+/bw	++/bw
12	+/bw	++/bw	+/bw	++/bw	+/bw	++/bw
13	-/bw	+/bw	-/bw	-/bw	-/bw	-/bw
51	+/b	++/bw	+/bw	++/bw	+/bw	++/bw
52	+/bw	++/bw	+/bw	++/bw	+/bw	++/bw
4	-/bw→b	+/bw→b	-/bw→b	+/bw→b	-/bw→b	+/bw→b
30	+/bw→b	+/bw→b	+/bw→b	+/bw→b	+/bw→b	+/bw→b
8	-/w	+/b	-/w	-/b	-/w	-/b
9	-/w	+/b	-/w	+/b	-/bw	+/b
14	-/w	+/b	-/w	-/b	-/w	-/b
15	-/w	+/b	-/b	-/b	-/w	-/b
19	-/bw	+/bw	-/bw	+/bw	-/bw	+/bw
23	-/w	+/b	-/w	+/b	-/bw	+/b
pGGA1/YEp <i>GPI8</i>	+/b	++/b	++/b	+++/b	+/b	+++/b
pSY1/YEp <i>GPI8</i>	+/h	++/h	++/h	+++/h	+/h	+++/h

The coloration of colonies was assessed as being dark blue (b), bluish (bw), or white (w). No clone with a blue halo (h), i.e. which secreted  $\alpha$ -gal/Ag $\alpha$ 1 was discovered. All cells grew normally at 24 °C and 37 °C on selective SDaa medium. Growth on SGalaa+X- $\alpha$ -Gal was assessed by eye and described relatively to the growth of positive control cells (W303-1B/pGGA1/YEp*GPI8*) on SDaa medium at 24 °C (+++= normal growth; ++= reduced growth; += slow growth; -= no growth). Clones 8, 9, 14, 15, 19 and 23 did not grow on SDaa+5-FOA, whereas clones above the triple line showed a phenotype depending on the presence of a YEp*gpi8\** plasmid (Table 2.)

1 of the remaining clones (clone 16) remained white after plasmid shuffling and apparently had lost the plasmid pGGA1 whereas the other 17 became dark blue as expected for wt  $\alpha$ -gal/Ag $\alpha$ 1 maturation. 6 out of these 17 clones (3, 5, 11, 18, 26 and 28) showed a phenotype for  $\alpha$ -gal/Ag $\alpha$ 1 maturation very similar to that of cells expressing wt *GPI8* and were discarded. Therefore, we were able to isolate totally 11 clones for which a reproducible, abnormal phenotype for  $\alpha$ -gal/Ag $\alpha$ 1 maturation or growth was proven to be due to the expression of a *gpi8\** allele (Table 2). It still might be the case that these isolated clones have a spontaneous mutations which reduce the number of plasmids they can support since for several clones, a negative growth phenotype and reduced coloration went together. However, these 11 clones together with the clones which did not grow on selective SDaa+5-FOA should be

subjected to analysis of the state of GPI anchor attachment including possible accumulation of complete GPI precursor glycolipids by labeling with [2-<sup>3</sup>H]-myo-inositol or retarded maturation of Gas1p by pulse-chase analysis. The summarized phenotype of all these 17 clones is listed in Table 1 (data of step VI).

**Table 2. Maturation of  $\alpha$ -gal/Ag $\alpha$ 1 on SGalaa+X- $\alpha$ -Gal (summary of step VI c)).**

clone	SGalaa+X- $\alpha$ -Gal 30 °C		SGalaa+X- $\alpha$ -Gal 30 °C (after SDaa+5-FOA) 20h
	20 h	4 d	
1	w	w	b
2	w	w	b
54	w	w	b
6	bw	bw	b
10	bw	bw	b
12	bw	bw	b
13	bw	bw	b
51	bw	bw	b
52	bw	bw	b
4	bw→b	bw→b	b
30	bw→b	bw→b	b
pGGA1/YEpGPI8	b	b	b
pSY1/YEpGPI8	h	h	h

The coloration of colonies was assessed as being dark blue (b), bluish (bw), or white (w). No clone with a blue halo (h), i.e. which secreted  $\alpha$ -gal/Ag $\alpha$ 1, was discovered.

### 3.2.3. Discussion

No *gpi8\** allele could be identified which encodes a proteolytic Gpi8p as estimated by forming a blue halo around colonies of wt cells expressing a GPI reporter protein and a random mutagenized *gpi8\** allele library. Of course, the reasons for this might be many-fold and a few possibilities are mentioned in the following: First, it is not possible to mutate Gpi8p in a way that it displays proteolytic activity in a living cell. (This does not exclude that Gpi8p might have been responsible for the hydrolysis of prominoPLAP as reported by Maxwell *et al.* (1995)). Second, we did not screen enough *gpi8\** alleles. Third, such a mutant would kill wt cells. This possibility cannot definitely be excluded by the fact that expression of the dominant-negative alleles C199A and C157A of *GPI8* did not affect growth if expressed from a YEpGPI8-type plasmid. Expression of C199A may cause accumulation of GPI precursors in the ER and this may serve as a signal to halt cell growth; on the other hand, cleavage of the GPI-SS and secretion of immature GPI proteins may result in a phenotype by which cell growth is not halted because GPI precursor proteins do not accumulate and cells grow with labile cell walls, so they simply would lyse in a hypotonic medium. Fourth, the amount of cleaved GPI reporter protein was too low to be detected by our system. Fifth, the cell might destroy putative GPI:protein transamidase complexes with mutant Gpi8p more rapidly than wt complexes or most of the cleaved  $\alpha$ -gal/Ag $\alpha$ 1 may rapidly be degraded within the cell since unprocessed  $\alpha$ -gal/Ag $\alpha$ 1 interacts with

the GPI anchoring machinery or other components of the yeast secretory pathway. 6<sup>th</sup>, overexpression of mutant Gpi8p was too low to see an effect. The C199A allele f.ex. had to be overexpressed at least 10 times in order to display a dominant-negative effect on growth of wt cells. Therefore, it might be interesting to know which effects are responsible for growth impairment by the selected *gpi8\** alleles. 7<sup>th</sup>, the screen was not designed to obtain maximal reproducibility. Positive clones might have been lost since the selection criteria were complicated through the fact that two multicopy vectors were present and thus may have generated a variable expression of both  $\alpha$ -gal/Ag $\alpha$ 1 and mutant Gpi8p. A more stable expression from genomically inserted constructs would have been more favorable. 8<sup>th</sup>, the “proteolytic” *gpi8* may act as dominant-negatives and simply be selected against so strongly, that they cannot be detected. It might have been better to express *gpi8\** alleles eventually also from an inducible promoter instead from the constitutive *GPI8* promoter.

However, with 27'000 clones more than 10 % of the total variability of the library was explored and led to the identification of dominant-negative alleles of *GPI8*. 11 clones (W303-1B/pGGA1/YEp*gpi8\**) or 0.041 % of totally 27'000 clones were selected to display an abnormal phenotype for the maturation of the GPI reporter protein  $\alpha$ -gal/Ag $\alpha$ 1. Normal maturation of protein  $\alpha$ -gal/Ag $\alpha$ 1 in these clones could be restored by shuffling out the YEp*gpi8\** indicating that the dominant-negative phenotype was due to expression of mutagenized *gpi8\** alleles. The clones can be subdivided into 3 groups, the first group which completely failed to export  $\alpha$ -gal/Ag $\alpha$ 1 (clones 1, 2 and 54); a second group with decreased expression of the GPI reporter protein on the cell surface as estimated by a reduced coloration of the cell wall (clones 6, 10, 12, 13, 51 and 52); and the third group of which export  $\alpha$ -gal/Ag $\alpha$ 1 was weakly impaired compared to cells expressing wt *GPI8* (Table 2). For all of these 11 clones, reduced growth rates were observed at least at 37 °C (Table 1). However, there is no evidence that this reduced growth is a direct consequence of impaired GPI anchoring activity in these cells, even more so, since it has to be found that all clones grew well on selective SDaa medium both at 24 and 37 °C at least for 3 days. Therefore, impaired growth appears to be an inaccurate or an invalid criterion to assess impairment of GPI anchor addition and might be due to several additive stress factors including carbon source substitution, temperature, expression levels of  $\alpha$ -gal/Ag $\alpha$ 1 and of *gpi8\** alleles, spontaneous mutations and so on. Several mutant clones (8, 9, 14, 15, 19 and 23) showed a strong negative growth phenotype on SGalaa+X- $\alpha$ -Gal which could not be assigned to the presence of a *gpi8\** allele. Expression of  $\alpha$ -gal/Ag $\alpha$ 1 on the surface of these cells appeared to be dependent on growth since under conditions where growth could be detected they became blue (Table 1).

The next step would be to confirm the results in new wt cells freshly transformed with plasmids extracted from the isolated clones. Then, GPI anchor attachment in all the 17 transformants should be analyzed by labeling with [2-<sup>3</sup>H]-myo-inositol to detect eventual accumulation of complete GPI precursors and by pulse-chase analysis to explore maturation of Gas1p. If possible this analysis should be performed by using cells which stably express both the GPI reporter protein and the mutant Gpi8p from an inducible promoter. *gpi8\** alleles which lead to a strong impairment of GPI anchoring efficiency should be sequenced, and might help to understand which structural features are required for proper function.

### 3.2.4. Experimental procedures

General information on strains, plasmids and protocols for the cultivation of yeast cells can be found in chapter 5.

#### *Plasmids*

Plasmids pGGA1 and pSY1 were kindly provided by M.P. Schreuder. pGGA1 corresponds to the published plasmid pGA1 (Schreuder *et al.*, 1993) but on which the phosphoglycerol kinase promoter (*PGKpr*) was replaced by the *GAL7* promoter between the restriction sites *EcoRI* and *SacI*. Plasmid YEp*gpi8*\* corresponds to plasmid YEp*GPI8* (Benghezal *et al.*, 1996) and bears instead of wt *GPI8* gene *gpi8* alleles generated by random PCR.

#### *Generation of the gpi8 allele library by random PCR*

For random PCR mutagenesis the protocol of Leung *et al.* (1989) was used to amplify a 1283 bp fragment of the *GPI8* ORF by using the primers *GPI-37u* (5'-CACATTT-AAGGCATAGCAA-3') and *GPI8XhoI* (5'-AAAAGTCTCGAGATTAGTGTACAGGTC-3'). The plasmid YEp*GPI8* (Benghezal *et al.*, 1996) was linearized by digestion with *AatII*, purified and used as template for PCR amplification. The YEp*gpi8*\* library containing mutant *gpi8* alleles was a ligation product of the 1074 bp *AlwNI/BstXI* fragment of the mutagenized PCR fragment and the 5791 bp *BstXI/XhoI* and 662 bp *XhoI/AlwNI* fragments of plasmid YEp*GPI8*. Conditions for the ligation were previously adapted to obtain maximal yield of correct ligation product which was then used for electroporation of XL1-Blue bacteria. The variability of the YEp*gpi8*\* library estimated by counting a part of the individual bacterial colonies and amounted to 260'000 different *gpi8* alleles.

### 3.2.5. Acknowledgements

I'm highly indebted to Markus Britschgi, who performed most of the selection procedure which was one of the topics of his diploma thesis. I thank Dr. M. P. Scheurer for the generous gift of  $\alpha$ -galactosidase expressing plasmids.

### **3.3. The GPI signal sequence of human placental alkaline phosphatase is not recognized by the yeast GPI anchoring machinery**

#### **3.3.1. Paper in preparation**

***The GPI signal sequence of human placental alkaline phosphatase is not recognized by the yeast GPI anchoring machinery +***

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**RUNNING TITLE:** *Specificity of the GPI transamidase*

**Key words:** Transamidase, Glycosylphosphatidylinositol, *Saccharomyces cerevisiae*, GPI anchoring signal, human placental alkaline phosphatase.

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**ABBREVIATIONS**

<sup>1</sup> The abbreviations used are: CRD = Cross Reacting Determinant; DHS = dihydrosphingosine; FOA = 5-fluoroorotic acid; GPI = glycosylphosphatidylinositol; GPI-SS = GPI signal sequence; hPLAP = human placental alkaline phosphatase; Ins = *myo*-inositol; wt = wild type.

### 3.3.2. Summary

Biosynthesis of glycosylphosphatidylinositol (GPI) anchored proteins involves the action of a GPI transamidase, which replaces the C-terminal GPI signal sequence (GPI-SS) of the primary translation product by a preformed GPI in the ER. The transamidation depends on two ER proteins, Gpi8p and Gaa1p. Although the GPI anchoring pathway is conserved throughout the eukaryotic kingdom, it has recently been reported that the GPI signal sequence of human placental alkaline phosphatase (hPLAP) is not recognized by the yeast transamidase but that it is recognized in yeast which contain the human Gpi8p homologue (De Sampaio *et al.*, 1999). This suggested to us, that Gpi8p is intimately implicated in the recognition process of GPI precursor proteins, which allows to discriminate them from those proteins that are not predestined to receive a GPI anchor. The report by De Sampaio demonstrated the presence of a GPI anchor on hPLAP only indirectly, namely by observing that, only in the presence of human Gpi8p the GPI anchored reporter gene was incorporated into cell membranes and the cell wall. Here we confirm that the GPI anchoring signal of hPLAP indeed is not recognized by the yeast GPI-anchoring machinery. However, in our hands GPI attachment cannot be restored by the co-expression of yeast/human Gpi8p fusion proteins in yeast cells under any circumstance. Yeast/human Gpi8p fusion proteins do not complement the lethality of  $\Delta gpi8$  cells and at least one construct of yeast/human Gpi8p displays a dominant-negative phenotype when overexpressed in yeast wild type cells. This suggests that the construct gets into the transamidase complex but nevertheless is not functional.

### 3.3.3. Introduction

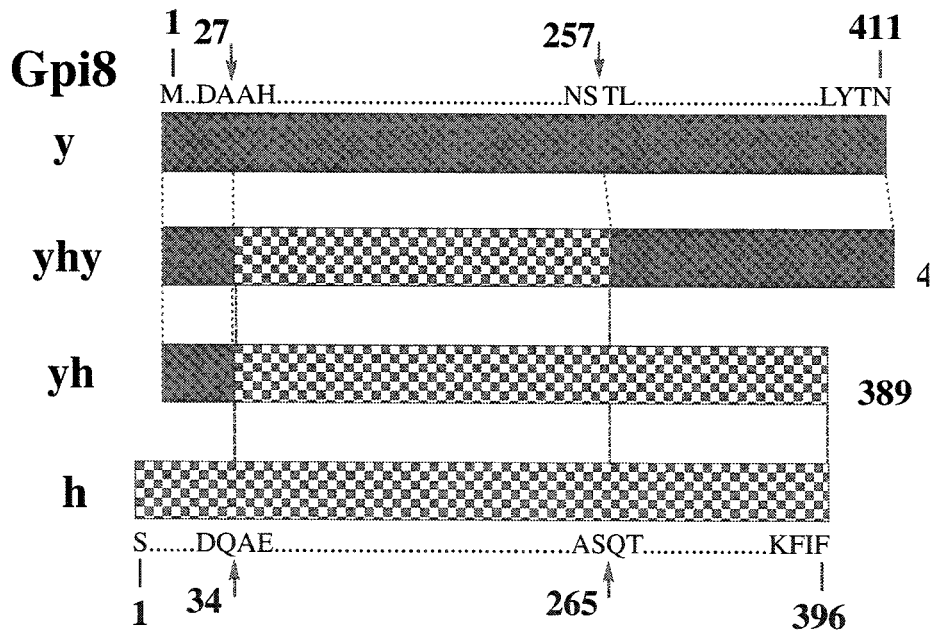
Membrane anchoring of cell surface proteins by means of glycosylphosphatidylinositol (GPI) anchors is ubiquitous among eucaryotes (Tiede *et al.*, 1999; Youl *et al.*, 1998; Ferguson, 1999). GPI precursor proteins invariably contain a cleavable N terminal signal sequence and a C terminal GPI signal sequence (GPI-SS) which directs GPI anchor attachment (Udenfriend and Kodukula, 1995a). While or shortly after the newly made protein has been translocated into the ER, it is recognized by the GPI transamidase, an enzyme which replaces the GPI-SS by a preformed GPI precursor glycolipid. Thus, it operates a relatively complex reaction, which requires two substrates and yields two products.

Genetic approaches have identified two genes which seem to be intimately involved in the transamidation reaction, *GAA1* and *GPI8* {Hamburger *et al.*, 1995; Benghezal *et al.*, 1995; Benghezal 1996}. Both of them are essential. *GAA1* encodes a 68 kD ER protein with a large luminal domain, several membrane spanning domains, and a cytosolic KKKXX ER-retrieval signal at the extreme C terminus. *GPI8* encodes a type I ER membrane protein. Gaa1p and Gpi8p have recently been shown to form a complex (Ohishi *et al.*, 2000). *GPI8* has 25–28% homology to several plant and invertebrate cysteine proteases and the active site Cys and His residues of the yeast and human Gpi8p have been identified by site-directed mutagenesis (Meyer *et al.*, 2000; Ohishi *et al.*, 2000). Therefore, Gpi8p has been suggested to be the catalytic subunit of the putative transamidase complex. Nevertheless, there is no direct proof for this assumption and it remains a major challenge to attribute precise tasks to these two proteins in the context of the transamidation reaction.

The C terminal GPI-SS's of various GPI precursor proteins have no primary sequence homology. They consist of an amino acid to which the GPI can be attached (the so-called the  $\omega$  site residue), a short hydrophilic spacer region and a hydrophobic domain at the extreme C terminus (Moran and Caras, 1991). Inspection of natural GPI-SSs and testing the functionality of systematically mutated GPI-SSs of different proteins belonging to a range of different organisms have shown that for the spacer region and the C terminal hydrophobic domain no specific amino acids but rather the overall physicochemical properties and their lengths determine functionality. In contrast, the  $\omega$  sites invariably contain either Ala, Asn, Asp, Cys, Gly or Ser (Eisenhaber *et al.*, 1998; Caro *et al.*, 1997) (Gerber *et al.*, 1992) (Kodukula *et al.*, 1993 (Moran *et al.*, 1991 (Nuoffer *et al.*, 1993). The data also show that in all species the  $\omega+1$  site can be any residue except Pro and that the  $\omega+2$  site requires an amino acid with a small side chain. Certain organisms have clear preferences with regard to the  $\omega$  site. For example, in natural proteins of *Saccharomyces cerevisiae*, the GPI anchor has so far only be found to be attached to Asn or Gly. When the yeast machinery is forced to use Ser, Ala, Asp and Cys, GPI anchors are added, but not at the same rate as to Asn and Gly (Nuoffer *et al.* 1993). Experiments in COS cells with fusion proteins containing the GPI-SS of the trypanosomal VSG protein have suggested that the requirements for GPI anchoring in mammalian cells and parasitic protozoa are similar but not identical (Moran and Caras, 1994), a result which has recently been qualified and refined by a study showing that COS cells very efficiently express trypanosomal VSG protein if only its  $\omega$ ,  $\omega+1$  and  $\omega+2$  sites are altered. (White *et al.*, 2000). Thus, the bulk of the studies points to the capital importance of the residues in the  $\omega$ ,  $\omega+1$  and  $\omega+2$ . (While data by White *et al.* argue that species preferences are due to preferences for amino acids in the the  $\omega$ ,  $\omega+1$  and  $\omega+2$  positons, the comparison with the report of Morgan and Caras leads one to realize that beyond these 3 amino acids, there has to be some



physico-chemical property or some unrecognized higher order structure that lends an explanation why one VSG protein is well recognized in COS cells whereas another VSG, having the same three amino acids at the  $\omega$ ,  $\omega+1$  and  $\omega+2$  sites, is not.). An other even more drastic species incompatibility has recently been reported by De Sampaio *et al.* (1999) in a study, whose primary purpose was to understand the features which allow a given GPI protein to stay at the plasma membrane whereas another protein is further processed and gets integrated into the cell wall (). The authors incidentally observed that the GPI-SS of hPLAP fused to the guar  $\alpha$ -galactosidase reporter protein was not recognized by the GPI anchoring machinery of *S. cerevisiae*, but that it was recognized if the human Gpi8p is coexpressed in these cells. It is this effect of human Gpi8p that started our present study, because it suggested that, within the transamidase, it was the Gpi8p subunit that dictated specificity and hence was involved in substrate recognition and in discriminating GPI proteins from other proteins. Thus, it attributed a specific function to one protein of the transamidase complex. The implication of this observation seemed important and so we decided to repeat the experiment of De Sampaio *et al.* and to carry out appropriate controls which ruled out, that the observed effect of *hGPI8* was not also observed with *yGPI8*, was due to strain differences etc. Our experiments document that yeast Gpi8p does not recognize the GPI-SS of hPLAP, that *hGPI8* can be expressed in yeast to high levels but that it has no effect on the processing of the GPI-SS of hPLAP.



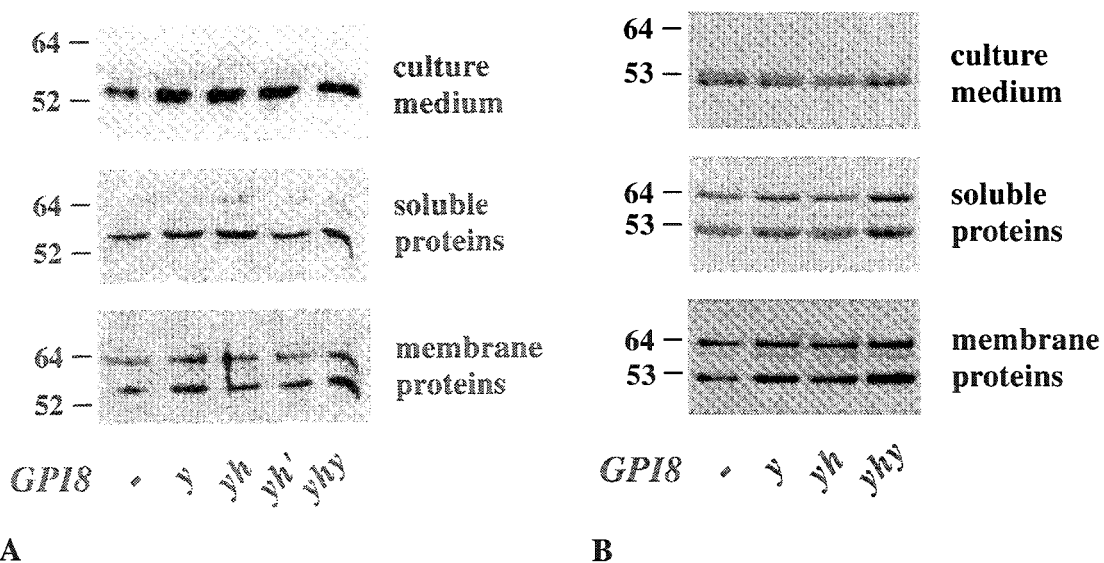
**Figure 1. The structure of yeast(y)/human(h) Gpi8p chimerae.**

The hybrid proteins yhGpi8p and yhyGpi8p both contain the first 27 amino acid residues of the yeast Gpi8p which are responsible for ER targeting and translocation. The putative cleavage site of the signal peptidase is between amino acids G<sup>21</sup> and A<sup>22</sup>. In yhGpi8p, the yeast N-terminal sequence is followed by the 362 C-terminal amino acids of the human protein starting with A<sup>35</sup>. In the yhyGpi8p, the yeast N-terminal sequence is attached to the 231 amino acids of the human protein from position 35 to 265 followed by the 154 C-terminal amino acids of the yeast protein.

### 3.3.4. Results

#### *Reinvestigation of the mechanism by which yhGpi8p rescues gpi7-1 gpi8-1 double mutants*

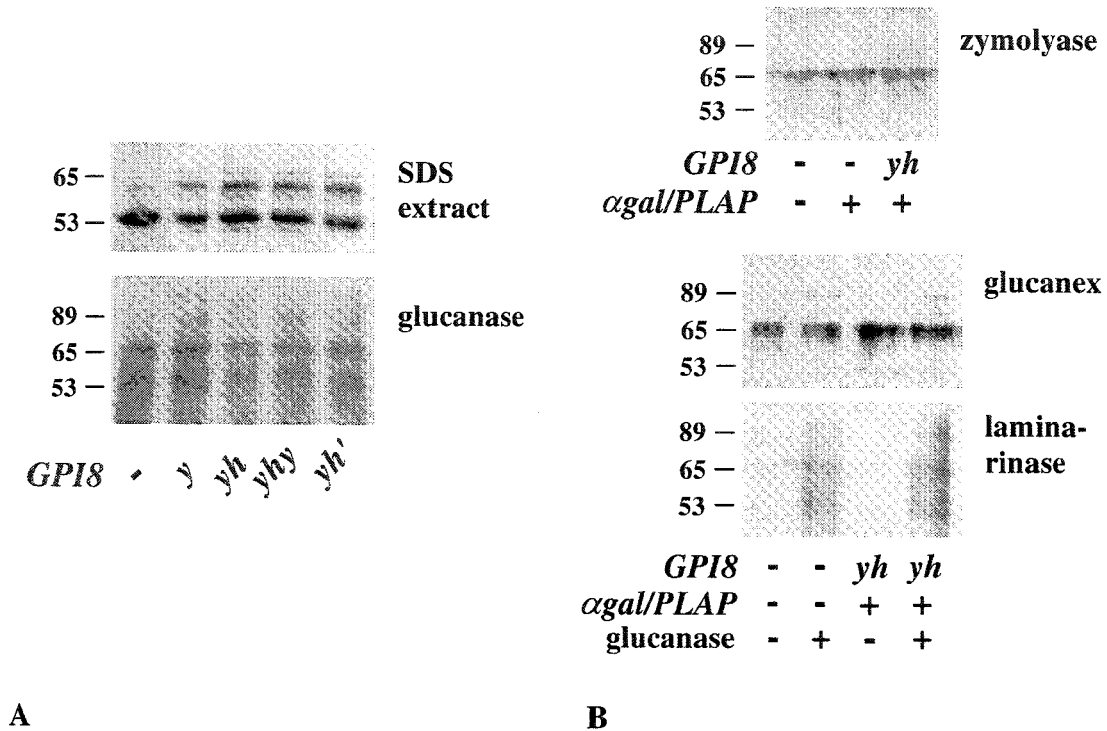
In our original report on Gpi8p we had observed that transfection of the yhGpi8p, containing the signal sequence plus 5 amino acids of yGpi8p followed by amino acids 35 to 396 of human Gpi8p corrected the temperature sensitive phenotype of *gpi7-1 gpi8-1* double mutants, thus suggesting that human Gpi8p is functional in yeast. However further studies showed that  $\Delta gpi8$  was not able to rescue  $\Delta gpi8$  cells, since diploid *GPI8/ $\Delta gpi8$*  cells containing yhGpi8p on a multicopy vector, when sporulated, only yielded 2 viable spores (unpublished). Furthermore, as shown in Table I,  $\Delta gpi8$  cells even were unable to grow without the plasmid born yGpi8p when yhGpi8p or yhyGpi8 (see below) were massively overexpressed from the *CUP1* promoter. (The inducible system was chosen to reduce negative selection against non-functional Gpi8p alleles (Meyer *et al.*, 2000)). Analysis of the original *gpi8-7-1 gpi8-1* that grew at 37°C after transfection of yhGpi8p showed that they had undergone a complicated recombination at the *GPI8* locus. Sequencing of the coding region of the genomic *GPI8* in *gpi8-1* showed no mutation (data not shown) but Gpi8p expression is reduced in *gpi8-1* mutants to about 50% of wild type (wt) levels at the restrictive temperature (Meyer *et al.*, 2000). These studies clearly show that human Gpi8p is not able to replace yGpi8p in yeast cells. We thus surmise that the original *gpi8-7-1 gpi8-1* that grew at 37°C after transfection of yhGpi8p were rescued because of a recombination of plasmid and genomic sequences, which restored normal expression of yGpi8p. The report by De Sampaio *et al.* (1999), demonstrating that the same yhGpi8p construct was able to recognize a human GPI anchoring signal thus suggested that our previous failure to rescue  $\Delta gpi8$  cells with yhGpi8p were due to the fact that this protein, although folded correctly, could not recognize the GPI anchoring signals of some essential yeast GPI proteins.



**Figure 2. Expression of yeast/human Gpi8p chimerae does not change the subcellular distribution of  $\alpha$ -gal/hPLAP.**

*gpi8-1* (A), or W303-1B (B) cells grown at 24 °C in SDaa medium lacking uracile and leucine constitutively expressed Gpi8 fusion proteins from YEp*GPI8*-type multicopy vectors.

Expression of  $\alpha$ -gal/hPLAP from plasmid pGS-PLAP was induced by transferring exponentially growing cells from SDaa to SGaa medium for 5 h at 37°C. Cells were harvested and subjected to subcellular fractionation as described by De Sampaio *et al.* (1999).  $\alpha$ -gal/hPLAP was detected by immunoblotting with anti- $\alpha$ -galactosidase antibodies. Numbers indicate molecular masses of protein standards in kD; yh': authentic *gpi8-1* strain with the yhGpi8p containing plasmid that was used by De Sampaio *et al.* (1999). The band of 64 kD is unspecific.



**Figure 3.  $\alpha$ -gal/hPLAP cannot be released from yeast cell walls by glucanase.**

**A)** Crude cell walls of *gpi8-1* from the very same experiment as described in Figure 2A were extracted twice with SDS and washed 15 times in 1 mM PMSF as described by De Sampaio *et al.* (1999). Extracted cell walls were digested with the glucanase called "laminarinase". **B)** Cell walls were prepared as described in **A**, but from *gpi8-1* overexpressing yhGpi8p from a genomically integrated copy under the control of the *GAL1-10* promoter (zymolyase and glucanex) or from a multicopy vector under the control of the yeast *GPI8* promoter (laminarinase). Cell walls were digested with various glucanases as indicated.  $\alpha$ -gal/hPLAP in the SDS extracts and glucanase digests of cell walls was detected by immunoblotting with anti- $\alpha$ -galactosidase antibodies. All clones transformed with pGS-PLAP expressed  $\alpha$ -gal/hPLAP efficiently and at the same level as assessed by immunoblotting the whole cell protein extracts (not shown); yh': clone used by Sampaio *et al.* (1999).

### *Co-expression of human Gpi8p does not alter subcellular distribution of $\alpha$ -gal/hPLAP*

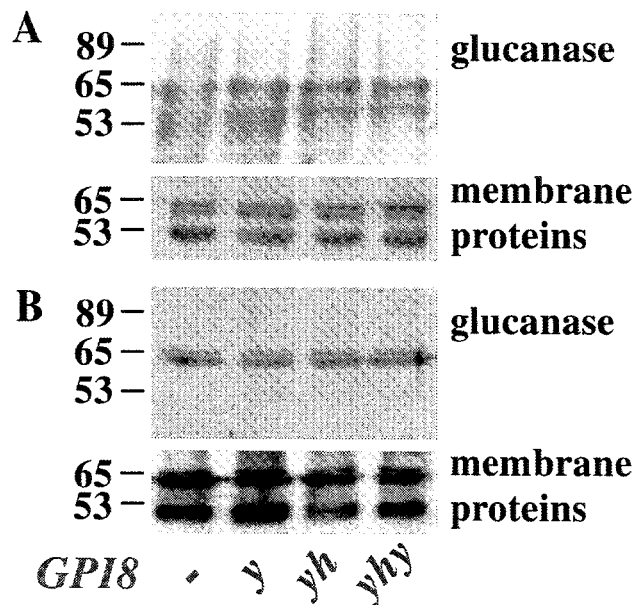
Previous studies had shown that GPI anchoring is a necessary to target proteins for integration into the cell wall by covalent attachment of the  $\alpha$ 1,4 linked mannose of GPI anchors to the  $\beta$ 1,6-glucan of the yeast cell wall (van der Vaart *et al.*, 1996). De Sampaio *et al.*, report that the translation product of a transfected reporter gene guar  $\alpha$ -galactosidase having at its C terminus the 39 amino acids-long GPI-SS hPLAP is found in the culture medium and the soluble fractions but absent from the membranes and the cell wall of wt yeast cells, but that the same reporter ( $\alpha$ -gal/hPLAP) is mainly found in cell membranes and in the cell wall, if it is expressed in *gpi8-1* cells which coexpress human Gpi8p (De Sampaio *et al.*, 1999). Although indirect, this drastic shift of the reporter from soluble fractions into membranes and into the cell wall strongly suggested that the protein had been GPI anchored in the *gpi8-1* cells. To reproduce this experiment we transformed *gpi8-1* cells with the identical multicopy vector harboring  $\alpha$ -gal/hPLAP under the control of the *GAL7* promoter as used by De Sampaio *et al.*, and in addition with YEp*GPI8*-type plasmids expressing yeast and yeast/human Gpi8p chimerae under the control of the yeast *GPI8* promoter (Fig.1 and Table 2B).

We tested whether co-expression of human Gpi8p's would lead to a different subcellular localization of  $\alpha$ -gal/hPLAP as compared to the appropriate control cells used by De Sampaio *et al.* (1999). Following the very same experimental procedures, our results show that subcellular distribution of  $\alpha$ -gal/ hPLAP was not dependent on the presence of yeast or yeast/human chimeric Gpi8 fusion proteins indicating that overexpression of Gpi8ps does not have any effect on the maturation of  $\alpha$ -gal/hPLAP (Fig. 2A). This observation was in strong contrast to the results published by De Sampaio *et al.* (1999) which had shown that - if yhGpi8p is co-expressed - only faint signals of  $\alpha$ -gal/PLAP were present in the culture medium and the soluble cell protein fraction. We therefore hypothesized that the restoring effect shown by De Sampaio *et al.* (1999) could have been due to a spontaneous mutation occurring either on the YEp*yhGPI8* plasmid, the  $\alpha$ -gal/hPLAP-expressing pGS-PLAP plasmid or within the genome of *gpi8-1*. However, repetition of the experiment with the very same transfectants used by De Sampaio *et al.* (1999) gave again no altered maturation of  $\alpha$ -gal/hPLAP in *yhGpi8* expressing cells compared to control cells (Fig. 2A); this excludes that the restoring effect of yhGpi8 on  $\alpha$ -gal/hPLAP maturation might have been due to genetic differences of cells or plasmids. We constructed and tested also an additional yeast/human chimera of *GPI8*, named *yhyGPI8* (Fig. 1), which only contains the highly conserved part of *hGPI8* including the active site residues (Meyer *et al.*, 2000; Ohishi *et al.*, 2000) whereas the the C terminal regions, including a luminal, non-conserved domain of about 80 amino acids plus the transmembrane and cytosolic domains are taken from yeast Gpi8p. Nevertheless, the *yhyGPI8* construct - as also shown for the *yhGPI8* construct - did not alter the subcellular distribution of  $\alpha$ -gal/hPLAP (Fig. 2A). Furthermore, subcellular distribution of  $\alpha$ -gal/hPLAP was also not affected by co-expression of yeast/human Gpi8p if cells were either incubated at 24 instead of 37 °C or if the induction of  $\alpha$ -gal/hPLAP was prolonged from 5 h up to 14 h or if the experiment was performed in wt cells instead of *gpi8-1* (Fig. 2B and data not shown).

In contrast to the findings of De Sampaio *et al.* (1999), wt cells contain the  $\alpha$ -gal/hPLAP in the crude membrane fraction as well and expression of yhGpi8p or yhyGpi8p do not increase the amount of reporter in the membrane fraction.

***$\alpha$ -gal/PLAP is not released from cell walls by glucanase digestion***

A further indication that a GPI protein underwent GPI anchor addition is its covalent integration into the yeast cell wall (van der Vaart *et al.*, 1996). We therefore isolated the yeast cell wall of our *gpi8-1* transformants and removed non-covalently attached proteins by two consecutive SDS extractions as performed by De Sampaio *et al.* (1999). Cell walls were treated with  $\beta$ -glucanase from different sources, namely laminarinase, zymolyase and glucanex (Fig. 3). As can be seen, no significant amounts of  $\alpha$ -gal/hPLAP could be released from yeast cell walls although longer exposure with anti- $\alpha$ -galactosidase antibodies showed several unspecific bands in the glucanase-treated fraction which were also detected in *gpi8-1* cells lacking  $\alpha$ -gal/hPLAP (Fig. 3B). The same result was also obtained in wt cells (data not shown).



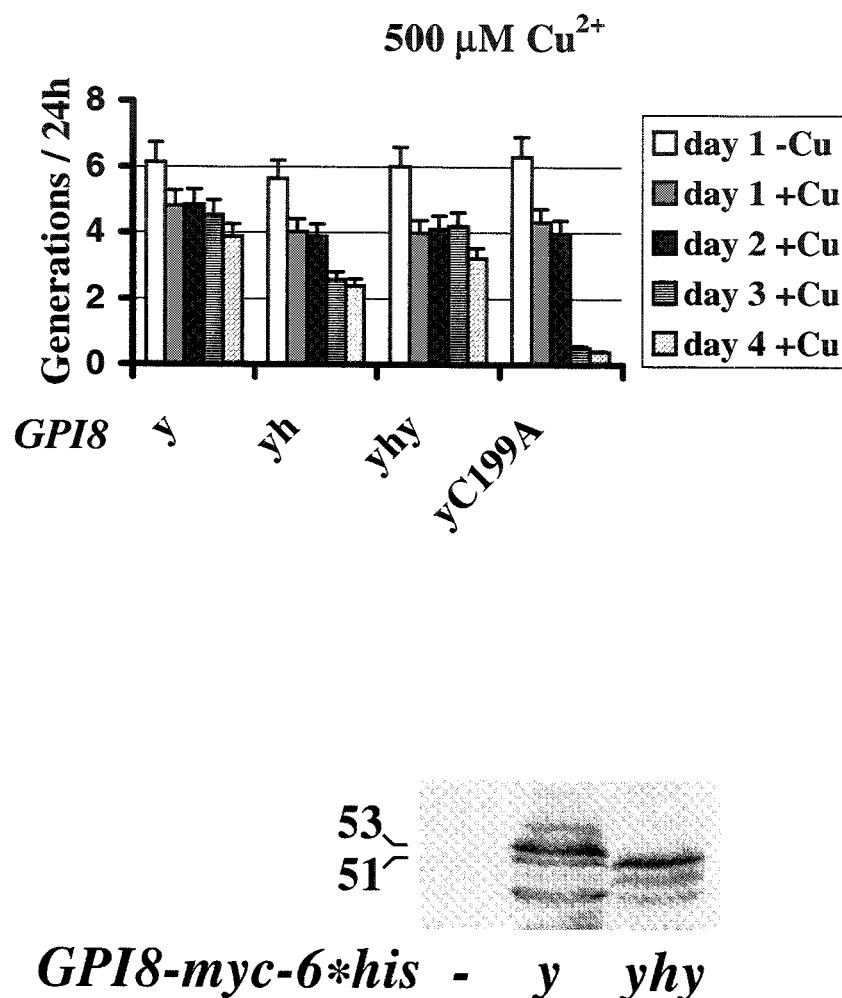
**Figure 4.  $\alpha$ -gal/hPLAP maturation is not altered by strong overexpression of yeast/human Gpi8p fusion proteins.**

A, *gpi8-1* cells were grown at 24 °C in SDaa medium lacking uracile and leucine to exponential phase. Simultaneous expression of  $\alpha$ -gal/hPLAP from plasmid pGS-PLAP and of yeast/human Gpi8p from constructs under the control of *GAL1-10* promoter and integrated into the genomic *URA3* locus was induced by shifting transformants from SDaa to SGaa medium and culturing them for 14 h at 37°C. B)  $\Delta gpi8/GAL1-10GPI8$  cells (FBY165) expressing  $\alpha$ -gal/hPLAP from the pGS-PLAP-type plasmid pBF518 under the hypoxic promoter *SRP1* and yeast/human Gpi8p from constructs on pBF54-type plasmids under the control of the strong *CUP1* promoter were grown at 24 °C to exponential phase in selective SGaa medium. Expression of genomically-inserted yeast *GPI8* was stopped by shifting cells to SDaa medium and cultures were incubated for 1h at 37 °C. Then, expression of yeast/human Gpi8p was induced by the addition of CuSO<sub>4</sub> to a final concentration of 200  $\mu$ M

and incubation was prolonged for 15 h under hypoxic conditions in order to express permanently  $\alpha$ -gal/hPLAP. Finally, cells were harvested and subjected to subcellular fractionation as described in Figures 2 and 3.  $\alpha$ -gal/hPLAP was detected by immunoblotting with anti- $\alpha$ -galactosidase antibodies. Numbers indicate molecular masses of protein standards in kD.

***Strong overexpression of human Gpi8p does not alter maturation of  $\alpha$ -gal/hPLAP***

Genetic evidence suggests that the GPI transamidase consists of a complex of several proteins (Meyer *et al.*, 2000). It seemed possible that the yhGpi8p and yhyGpi8p were inactive since their affinity for the other components of the complex was lower than the one of the endogenous yGpi8p. We therefore overexpressed chimeric *GPI8* under the control of the galactose inducible *GALI-10* promoter integrated into the genomic *URA3* locus or under the control of the strong *CUP1* promoter from a single copy vector as well as from a multicopy vector. Previous experiments had shown that these promoters achieved expression levels of approximately 12, 15 and 80 times the amount of endogenous yGpi8p, respectively. In addition, we increased the time of  $\alpha$ -gal/hPLAP induction on galactose from 5 to 14 h in order to make sure that cells took up normal growth rates since we had observed cells needed at least 3 to 6 hours – depending on the strain – to resume normal growth rates after a shift from glucose to galactose. After a 14 h induction in galactose all transformants had undergone 2 to 3 generations, grew as rapidly as wt cells, and displayed very high and identical expression levels of  $\alpha$ -gal/hPLAP (data not shown). Nevertheless the subcellular distribution of the reporter was the same in all cases, no improved maturation of  $\alpha$ -gal/hPLAP was obtained as shown for the membrane protein extract and the glucanase-extracted cell wall proteins (Fig. 4A). To force even more the incorporation of human Gpi8p into the transamidase complex, we overexpressed human Gpi8ps in cells which concomitantly were depleted of the endogenous yGpi8p but even this did not affect the subcellular localization of  $\alpha$ -gal/hPLAP. We also argued that the subcellular fractionation procedure described by De Sampaio *et al.* (1999) might not be efficient enough to see differences in subcellular distribution of  $\alpha$ -gal/hPLAP when it was strongly expressed. We therefore attempted to improve separation by partitioning membrane into the TX-114 phase (Bordier, 1981; Fankhauser and Conzelmann, 1991) or by using ultracentrifugation (Feldheim and Schekman, 1994). Again, these did not reveal altered subcellular distribution of the reporter when yhGpi8p chimerae were expressed (data not shown). All these results show that expression of human Gpi8p does neither increase nor decrease the incorporation of  $\alpha$ -gal/hPLAP into membranes and into the cell wall.



**Figure 5. Expression of yhGpi8 has a partial dominant-negative effect on cell growth of W303 cells.**

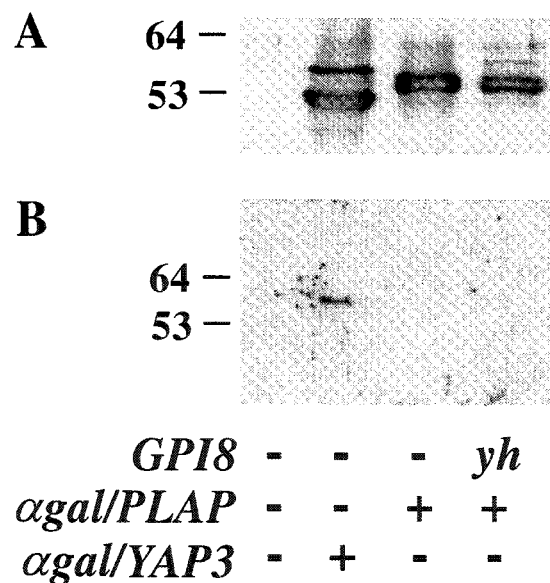
**A.** Hybrid genes under the control of the copper-inducible *CUP1* promoter were expressed from the centromeric pBF54-type vectors pBF516 and pBF517. W303 cells, harboring yeast (y), yeast-human hybrid (yh, yhy) or the yeast dominant-negative active site mutant of *GPI8* (yC199A; (Meyer *et al.*, 2000)) were grown to exponential phase at 24 °C without Cu<sup>2+</sup> (0.75-1.0  $\mu$ M Cu<sup>2+</sup>) in SDaa medium lacking Trp. Cells were resuspended at 0.2-0.3 OD<sub>600</sub>/mL in fresh normal (-Cu) or CuSO<sub>4</sub>-containing (+Cu) SDaa medium. After 1 h at 24 °C, cultures were shifted to 37 °C. Cell growth was monitored in triplicate cultures at short intervals by densitometry during 1-4 days. Cells were diluted each time they attained 2.0 OD<sub>600</sub>. The number of cell divisions during successive periods of 24 h are represented by columns. **B.** Myc-His6 tagged yGpi8p and yhyGpi8p were highly overexpressed from plasmids pBF519 and pBF520, respectively. W303-1B transformants were cultured as described in A and harvested 3 h after the addition of Cu<sup>2+</sup>. Proteins in the whole cell extract

were detected by immunoblotting with anti-c-myc antiserum. Molecular weight differences between yGpi8p and yhyGpi8p might be explained by different glycosylation patterns. Yeast Gpi8p has 3 N glycosylation sites, hGpi8p has no N glycosylation site predicted (Benghezal *et al.*, 1996).

***yhGpi8p interferes with the yeast GPI anchoring machinery***

We tried to investigate whether yhGpi8p had any dominant negative effect on cell growth, a finding that would indicate that yhGpi8p competes with endogenous yGpi8p for integration into the transamidase complex but is not properly functioning in this complex. (Meyer *et al.*, 2000). We therefore checked whether our Gpi8p chimeras affected cell growth of wt cells. yhGpi8ps were expressed under the control of the *GALI-10* promoter from genomically integrated constructs. None of the constructs had any growth retarding effect on W303 (data not shown). However, when we expressed our fusion proteins under the control of the stronger *CUP1* promoter from a single copy vector, cell growth of W303 was significantly retarded by the yhGpi8 chimeric protein indicating that the yhGpi8p interferes with the yeast GPI anchoring machinery (Fig. 5A). On the other hand, no significant growth retardation was obtained with the yhyGpi8 fusion protein even though it was strongly overexpressed. (To show strong overexpression of yhyGpi8p, we had to tag yGpi8p and yhyGpi8p since our antibodies made against a fragment of yGpi8p only poorly recognize the human homologue. As documented by Fig. 5B, using tagged versions, yhyGpi8p can be overexpressed to the same degree as yGpi8p. Thus the failure to see any dominant negative effect of yhyGpi8p does not seem to be due to poor expression of this allele.)

We further investigated whether  $\alpha$ -gal/hPLAP expression somehow impairs GPI anchoring in yeast cells. For that, we analyzed the maturation of the GPI-anchored protein Gas1p by immunoblotting. Overexpression of yeast human Gpi8p fusion proteins did not significantly affect Gas1p maturation (data not shown).



**Figure 6.  $\alpha$ -gal/hPLAP is not GPI-anchored.**

*gpi8-1* transformants were grown at 24 °C in selective SDaa medium. Expression of  $\alpha$ -gal fusion proteins was induced by transferring exponentially growing cells from SDaa to SGaa



medium, wherein they were cultured for 14 h at 24 °C.  $\alpha$ -gal/hPLAP was expressed from plasmid pGS-PLAP and  $\alpha$ -gal/YAP3 from plasmid pGS-YAP3. Overexpression of yGpi8p from plasmid pBF516 was induced by adding CuSO<sub>4</sub> (200  $\mu$ M) 3 h before harvesting cells. Cells were subjected to rapid protein extraction followed by immunoblotting with anti- $\alpha$ -galactosidase antibodies (panel A) or labeling with [2-<sup>3</sup>H]Ins/[4,5-<sup>3</sup>H]DHS followed by immunoprecipitation with anti- $\alpha$ -galactosidase antibodies, SDS-PAGE and detection by fluorography (panel B) as described in Experimental Procedures. Numbers indicate molecular masses of protein standards in kD.

***$\alpha$ -gal/hPLAP is not recognized by the yeast GPI anchoring machinery***

De Sampaio *et al.*, had reported that  $\alpha$ -gal/hPLAP was not membrane associated in wt cells and taken the membrane association of  $\alpha$ -gal/hPLAP in cells expressing heterologous Gpi8p alleles as an indication, that the protein had been modified by the addition of a GPI anchor. Since in our hands part of  $\alpha$ -gal/hPLAP was membrane associated in wt we decided to reinvestigate whether  $\alpha$ -gal/hPLAP is recognized by the yeast GPI anchoring machinery using a more direct approach. We therefore transformed *gpi8-1* cells with pGS-PLAP and with the plasmids pBF54 and pBF516 overexpressing yGpi8p and yHpi8p, respectively. As a control, we transformed *gpi8-1* with the plasmid pGS-YAP3, a pGS-PLAP-type plasmid on which the 39 amino acids long GPI-SS of human PLAP was replaced by the 35 amino acids long GPI-SS of yeast Yap3p. The whole cell protein extract of all these transformants were first analyzed by immunoblotting. Anti- $\alpha$ -galactosidase showed mainly the major 54 kD band in cells containing  $\alpha$ -gal/hPLAP whereas two bands were observed in cells containing  $\alpha$ -gal/YAP3 (Fig. 6A). The upper 56 kD band of  $\alpha$ -gal/YAP3 was observed in several independent experiments and suggested a different maturation of  $\alpha$ -gal/YAP3 with respect to  $\alpha$ -gal/hPLAP. Therefore, we subjected these *gpi8-1* transformants to labeling with tritiated *myo*-inositol and dihydrosphingosine which both are specifically incorporated into GPI anchors.  $\alpha$ -gal/hPLAP was precipitated with anti- $\alpha$ -galactosidase antibodies and detected by fluorography. As can be seen in Figure 6B, only  $\alpha$ -galactosidase fused to the GPI-SS of yeast Yap3p could be radiolabeled, whereas  $\alpha$ -galactosidase fused to the GPI-SS sequence of hPLAP was not radiolabeled. Furthermore, comparison of molecular weights indicates that only the upper, minor band of  $\alpha$ -gal/YAP3 is labeled and hence GPI anchored. Thus, it appears that by this criterium, that  $\alpha$ -gal/hPLAP is not recognized by the transamidase and that the problem resides within the GPI anchor signal of hPLAP since the same reporter is anchored when fused to the GPIanchoring signal of YAP3. For further confirmation we isolated the membrane proteins of all our *gpi8-1*, W303-1B and  $\Delta$ *gpi8* (FBY143) transformants, treated them with PI-PLC and analyzed anti- $\alpha$ -galactosidase immunoprecipitates or whole cell extracts by immunoblotting with anti-CRD antibodies. In agreement with the labeling experiment, we could not detect  $\alpha$ -gal/PLAP by anti-CRD antibodies after PI-PLC treatment although GPI-anchored proteins such as Gas1p were easily detectable (data not shown). Thus, the data indicates that the GPI-SS of human PLAP was not recognized by the GPI anchoring machinery and that this recognition cannot be restored by overexpressing yHpi8p. We therefore suppose that other factors than Gpi8p are involved in GPI-SS recognition by the putative GPI:protein transamidase. However, we cannot exclude

that minor amounts of  $\alpha$ -gal/hPLAP were GPI-anchored and remained below the detection limit of our assay.

### 3.3.5. Discussion

Signal sequences for GPI anchor addition appear to be structurally similar among eucaryotic GPI proteins (Udenfriend and Kodukula, 1995a). However, GPI-SSs do not show a consensus sequence and are at least to some extent organism-specific (Moran and Caras, 1994; Takos *et al.*, 2000). GPI-SSs of diverse origins have been described to differ among each other mainly in their overall length and amino acid sequence close of the  $\omega$  site region (Eisenhaber *et al.*, 1998), also called the small amino acid domain (SAD; (Aceto *et al.*, 1999)); nevertheless, precise organism-specific features were not described in detail up to now. Even less is known about the specific recognition of the GPI-SS by the GPI anchoring machinery of a defined organism. Constraints for the GPI-SS binding site of the GPI:protein transamidase were calculated by computational analysis and predicted to include the recognition of a flexible, unstructured region of approximately 11 amino acids upstream of the  $\omega$  site in the GPI-SS (Eisenhaber *et al.*, 1998). Regardless, it can be assumed that the catalytic subunit of the transamidase complex is involved in GPI-SS recognition. Indeed, it has been recently reported that the human GPI-SS of placental alkaline phosphatase is only recognized by the yeast GPI anchoring machinery if human Gpi8p, suggested to be the catalytic subunit of the transamidase (Benghezal *et al.*, 1996), is co-expressed (De Sampaio *et al.*, 1999). As distinct from the results published by De Sampaio *et al.* (1999), we show that co-expression of human Gpi8p does not restore GPI anchor addition onto the  $\alpha$ gal/PLAP fusion protein which is not recognized by the yeast GPI anchoring machinery. Co-expression of the yhGpi8p as used by De Sampaio *et al.* (1999) and an additional yeast/human Gpi8p chimerae (yhyGpi8p) did not lead to an altered subcellular distribution of the  $\alpha$ gal/PLAP protein. Independent on the expression level of yeast/human Gpi8p chimerae,  $\alpha$ gal/PLAP was present in the membrane protein as well as in the soluble protein fraction; moreover,  $\alpha$ gal/PLAP was present also in the culture medium of all tested transformants. This shows that  $\alpha$ gal/PLAP was mislocalized, even at lower expression levels. Furthermore, we could not release significant amounts of  $\alpha$ gal/PLAP from SDS-extracted cell walls by glucanase digestion indicating that  $\alpha$ gal/PLAP was not covalently incorporated into yeast cell walls. Therefore we analyzed whether  $\alpha$ gal/PLAP became modified by a GPI-anchor. Labeling with tritiated *myo*-inositol and dihydrosphingosine demonstrates that the  $\alpha$ -galactosidase reporter protein is only GPI-anchored if it is fused to the GPI-SS of Yap3p but not when fused to that of human PLAP. This indicates that the GPI-SS of hPLAP is not recognized by the yeast GPI anchoring machinery and in addition, its recognition cannot be restored by human Gpi8p. We explain the contradictory results of De Sampaio *et al.* (1999) by both strain differences and different expression levels of  $\alpha$ gal/PLAP without knowing the precise reasons. Both yeast/human *GPI8* constructs described in this study were not able to complement the lethality of a yeast *gpi8* deletant (Table 1). In mammalian cells, it has been shown that yeast Gpi8p can complement a human *gpi8* deletant if the region from position 298 to position 284 is substituted by the corresponding human sequence indicating specificity of the yeast Gpi8p and human Gpi8p is concentrated onto this region (Ohishi *et al.*, 2000). However, one of our yeast/human chimerae (yhGpi8p) showed a dominant-negative effect on growth of wt cells. This suggests - as compared to studies with yeast *gpi8* active site mutants (Meyer *et al.*, 2000) - that yhGpi8p interferes with the yeast GPI

anchoring machinery. In addition, the reporter protein  $\alpha$ -galactosidase does not impede recognition of  $\alpha$ gal/PLAP by the yeast GPI:protein transamidase since  $\alpha$ gal/YAP3 was efficiently GPI-anchored. Altogether, these results might be an indication that other factors in addition to Gpi8p might be involved in GPI-SS recognition. One possible candidate could be Gaalp, another subunit of the GPI:protein transamidase (Ohishi *et al.*, 2000), since overexpression of yeast Gaalp, but not of yeast Gpi8p, could partially suppress the impaired processing of GPI-SS mutants of Gas1p (Hamburger *et al.*, 1995; chapter 3.1.2.1.).

**Table 1** Complementation of the Growth Defect of  $\Delta$ *gpi8*<sup>a</sup>.

transfected hybrid <i>GPI8</i> gene	Growth on FOA	
	-Cu <sup>2+</sup>	+Cu <sup>2+</sup>
empty vector	-	-
<i>yGPI8</i>	+	+
<i>yhGPI8</i>	-	-
<i>yhyGPI8</i>	-	-

<sup>a</sup> Complementation of  $\Delta$ *gpi8* cells by yeast/human chimeric *GPI8* was assessed by plasmid shuffling. The haploid strain FBY525 ( $\Delta$ *gpi8*/YE*pGPI8*) was transformed with the single copy vectors pBF54 (*yGPI8*), pBF516 (*yhGPI8*), pBF517 (*yhyGPI8*) or the empty vector YCplac22. 12 to 16 clones from each transfected strain were streaked out on FOA containing plates to see if the wt *yGPI8* (on YE*pGPI8*) could be forced out. Only *yGPI8* could restore the growth defect of  $\Delta$ *gpi8* cells.

### 3.3.6. Acknowledgements

We are grateful to Drs Hans Kapteyn and Stephan te Heesen for the generous gift of reagents and strains. We thank Jean-Paul Bourdineuad for plasmids and strains used from the original studies (De Sampaio *et al.*, 1999). We are greatly indebted to Christine Vionnet for her excellent technical assistance. This work was supported by a grant No 3100-049664.96 from the Swiss National Foundation for Scientific Research.

### 3.3.7. Strains, Media and Materials.

*Saccharomyces* strains are listed in Table 2A and were grown in SD medium (Sherman, 1991) supplemented with all 20 amino acids (20-400 mg/L), adenine sulfate, and uracile (SDaa medium). SGaa medium is the same with 2% of galactose instead of glucose. The absorbance of dilute cell suspensions was measured at 600 nm, one OD<sub>600</sub> unit of cells corresponding to  $1.0\text{--}2.5 \times 10^7$  cells. The copper concentration of normal SDaa media was determined to be 0.75-1.0  $\mu$ M (Watkins *et al.*, 1971). Radiochemicals and materials were from the same sources as described (Meyer *et al.* 2000) or obtained from the following: Polyclonal anti-Cross Reacting Determinant (CRD) rabbit antibodies from Oxford GlycoSciences; anti-mouse and anti-rabbit IgG-peroxidase conjugates from Sigma. Polyclonal rabbit antibodies against guar  $\alpha$ -galactosidase were the kind gift of Dr. Hans Kapteyn (BioCentrum

Amsterdam, Netherlands). Anti-Gas1p (Fankhauser and Conzelmann, 1991) and affinity-purified anti-Gpi8p (Benghezal *et al.*, 1996) rabbit antibodies have been described previously. Oligonucleotide synthesis and DNA sequencing services were provided by MICROSYNTH, Balgach, Switzerland.

### Plasmids

Plasmids used in this study are listed in Table 2B and were constructed using standard procedures (Sambrook *et al.*, 1989). All inserts were verified by sequencing.

### Construction of plasmids containing Yeast/Human *GPI8* Chimerae.

The structure of the yeast/human chimerae are depicted in Figure 1. The plasmid YEp $hyGPI8$  was constructed by introducing an EcoO109I site by mutating a silent site of YEp $yhGPI8$  (Benghezal *et al.*, 1996) by PCR using the primers 5'-ACTTTGCAGGACCTCTTCCAGGTATGTCCCAAAAGTCTG-3' and 5'-TCGAA-GAGGTCCTGCAAAGTTAACGTGCTAGCTGGGTAAATTTCTTC-3'. The two PCR products were digested with EcoO109I/NdeI (362 bp) and EcoO109I/NdeI (77 bp), respectively, and were ligated with the PflmI/NdeI fragments of YEp $yhGPI8$  (5343 and 1231 bp) resulting in plasmid YEp $yhGPI8\_EcoO109I$ . YEp $hyGPI8$  was a ligation product of the 930 bp EcoO109I/SphI fragment of YEp $GPI8$  with the YEp $yhGPI8\_EcoO109I$  fragments PstI/EcoO109I, AatII/PstI and AatII/SphI of 708, 2922 and 2969 bp, respectively.

**Construction of YEp $yhGPI8_{yter}$ .** A SphI site was introduced into the terminator sequence of  $yGPI8$  on the YEp $GPI8$  vector by PCR using primers SphITER 5'-GACTAGTGCATGCAAC-3' and M13F 5'-TGTAACGACGGCCAGT-3'. The SphI/SphI-digested PCR fragment of 484 bp was then ligated with the SphI-linearized YEp $yhGPI8$  plasmid resulting in plasmid YEp $yhGPI8_{ter}$  harboring the  $yhGPI8$  gene with the yeast terminator sequence starting 25 bp downstream of the stop codon. *URA3*-based integrative plasmids containing the yeast/human *GPI8* chimerae under the control of the galactose-inducible *GAL1-10* promoter were constructed by ligating the 4813 bp SalI/AatII and the 832 bp AatII/AlwNI fragments of YIp $GALGPI8$  with the 1594 bp AlwNI/SalI fragment of YEp $yhGPI8_{yter}$  or with the 1632 bp AlwNI fragment of YEp $yhGPI8$  resulting in pBF513 and pBF514, respectively. Plasmids pBF516 and pBF517 harbor the  $yhGPI8_{yter}$  and the  $yhyGPI8$  hybrid genes under the control of the *CUP1* promoter on a centromeric vector. pBF516 was a ligation product of the 4057 bp SmaI/XbaI and the 286 bp XbaI/AlwNI fragments of pBF54 and the 1655 bp AlwNI/PshAI fragment of pBF513; pBF517 was obtained by ligating the 5929 bp SalI/BseRI fragment of pBF516 with the 1296 bp BseRI/SalI fragment of YEp $hyGPI8$ .

**Construction of myc-his6-tagged forms of *GPI8*.** A XhoI site was introduced by PCR at the end of *GPI8*. The primers *GPI8*-forwards37 5'-cacatttaagcatagcaaa-3' starting 37 nucleotides upstream of the starting ATG and *GPI8*-reverse12 (5'-aaaagtctcgagattagtgtacaggtc-3') ending 12 bp upstream of the stop codon were used to generate a 1.28 kb fragment which was digested with BstXI and XhoI. The resulting 86 bp fragment plus the 1.8 kb SacI/BstXI fragment of YEp $GPI8$  were ligated into the plasmid YEp(*ALG5His6*) (*URA3*, 2 $\mu$ ; obtained from Stephan te Heesen and Markus Aebi, unpublished) digested with SacI and XhoI. The resulting plasmid, YEp $GPI8$ -myc-his6, encodes a 430 amino acid full length Gpi8p which is myc-his6 tagged at its

C with the amino acids LEQKLISEEDLNHHHHHHN-COO<sup>-</sup>. Expression of Gpi8p-myc-his6 using YEp*GPI8-myc-his6* suppressed the temperature-sensitive growth defect of *gpi7 gpi8* (FBY122) and the lethality of  $\Delta$ *gpi8* (FBY525) indicating that the recombinant protein was active. The plasmids pBF519 and pBF520 express the full-length yeast *GPI8* and the chimeric *yhyGPI8* gene, respectively, marked at their C terminus by a *myc-his6* tag under the control of the *CUP1* promoter. pBF519 was obtained by ligating the 6416 bp BstBI/SalI fragment of pBF55 (Meyer *et al.*, 2000) with the 1159 bp BstBI/SalI fragment of YEp*GPI8-myc-his6*; pBF520 by ligating the 5796 bp BstXI/SphI fragment with the 1782 bp SphI/BstXI fragment of pBF519.

**Plasmids expressing the  $\alpha$ -galactosidase reporter fused to the C terminal GPI-SS of *hPLAP* and of yeast *YAP3*.** Plasmids pGS-PLAP, pGS12, pGS-YAP3 and pJPB21 were a kind gift of Dr Jean-Paul Bourdineaud and were described previously (De Sampaio *et al.*, 1999; Bourdineaud *et al.*, 1998). pBF518 expresses the  $\alpha$ -gal/hPLAP fusion protein under the control of the hypoxic *SRP1* promoter and was obtained by replacing the *GAL7* promoter-containing EcoRI/EagI fragment of pGS-PLAP by the 831 bp EcoRI/EagI fragment of plasmid pJPB21. Yeast strains were transformed by electroporation (Becker and Guarente, 1991).

#### Subcellular fractionation, protein extraction and Western Blot Analysis.

Cells were broken with glass beads and subcellular fractionation was performed as described previously (De Sampaio *et al.*, 1999). Whole cell protein extracts were obtained by the rapid protein extraction protocol (Horvath and Riezman, 1994). Proteins were separated by SDS-PAGE in 10% gels for  $\alpha$ -gal/hPLAP,  $\alpha$ -gal/Yap3p and Gpi8p, and 7.5 % gels for Gas1p (Laemmli, 1970). Western blots were developed by enhanced chemiluminescence (ECL Kit, Amersham, Buckinghamshire, UK).

**Table 2A Strains used in this study**

strain	genotype	plasmid
W303-1B	<i>MAT<math>\alpha</math> ade2-1 can1-100ura3-1 leu2-3,112 trp1-1 his3-11,15</i>	
FBY11	<i>MAT<math>\alpha</math> ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1</i>	
FBY165	<i>MAT<math>\alpha</math> ade2-1 leu2-3,112 his3-11,15 trp1-1 <math>\Delta</math>gpi8::kanMX2</i> <i>ura3-1::URA3-GAL1-10-GPI8</i>	
FBY525	<i>MAT<math>\alpha</math> ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 lys<sup>-</sup></i> <i><math>\Delta</math>gpi8::kanMX2</i>	YEp <i>GPI8</i>
FBY526	FBY525	pBF53
FBY577	W303-1B; <i>ura3-1::URA3-GAL1-10-GPI8-myc-his6</i>	
FBY580	W303-1B; <i>ura3-1::URA3-GAL1-10-yhGPI8</i>	
FBY581	W303-1B; <i>ura3-1::URA3-GAL1-10-yhyGPI8</i>	
FBY582	W303-1B; <i>ura3-1::URA3-GAL1-10-yhGPI8</i>	pGS-PLAP
FBY583	W303-1B; <i>ura3-1::URA3-GAL1-10-yhyGPI8</i>	pGS-PLAP
FBY584	FBY11; <i>ura3-1::URA3-GAL1-10-yhGPI8</i>	pGS-PLAP
FBY585	FBY11; <i>ura3-1::URA3-GAL1-10-yhyGPI8</i>	pGS-PLAP

**Table 2B Plasmids used in this study**

plasmid	description	reference
YCplac22	<i>TRP1 CEN4-ARS1 Ap<sup>R</sup></i>	Gietz and Sugino (1988)
YEplac112	<i>TRP1 2μ Ap<sup>R</sup></i>	Gietz and Sugino (1988)
YEp352	<i>URA3 2μ Ap<sup>R</sup></i>	??(Hill <i>et al.</i> , 1986)Hill <i>et al.</i> (1986)
YEpGPI8	YEp352; <i>GPI8</i>	Benghezal <i>et al.</i> (1996)
YEp <sub>yh</sub> GPI8	YEp352; <i>yhGPI8</i>	Benghezal <i>et al.</i> (1996)
YEp <sub>yh</sub> GPI8 <sub>tyter</sub>	YEp352; <i>yhGPI8<sub>tyter</sub></i>	this study
YEp <sub>hy</sub> GPI8	YEp352; <i>GPI8</i>	this study
YIpGALGPI8	YIpGAL; <i>GAL1-10-GPI8</i>	Meyer <i>et al.</i> (2000)
pGS-PLAP	<i>LEU2 2μ Ap<sup>R</sup>; GAL7-αGALPLAP</i>	De Sampaio <i>et al.</i> (1999)
pGS-YAP3	<i>LEU2 2μ Ap<sup>R</sup>; GAL7-αGALPLAP</i>	De Sampaio <i>et al.</i> (1999); and this study
pBF518	<i>LEU2 2μ Ap<sup>R</sup>; SRP1-αGALPLAP</i>	this study
pBF513	YIpGAL; <i>GAL1-10-yhGPI8</i>	this study
pBF514	YIpGAL; <i>GAL1-10-yhyGPI8</i>	this study
pBF513	YIpGAL; <i>GAL1-10-yhGPI8<sub>tyter</sub></i>	this study
pBF516	YCplac22; <i>CUP1-yhGPI8</i>	this study
pBF517	YCplac22; <i>CUP1-yhyGPI8</i>	this study
pBF519	YEplac112; <i>CUP1-GPI8-myc-his6</i>	this study
pBF520	YEplac112; <i>CUP1-yhyGPI8-myc-his6</i>	this study
pBF54	YCplac22; <i>CUP1-GPI8</i>	Meyer <i>et al.</i> (2000)
pBF55	YEplac112; <i>CUP1-GPI8</i>	Meyer <i>et al.</i> (2000)

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### 3.4. Multicopy suppressor screen with wt cells overexpressing the dominant-negative *gpi8* allele C199A

#### 3.4.1. Introduction

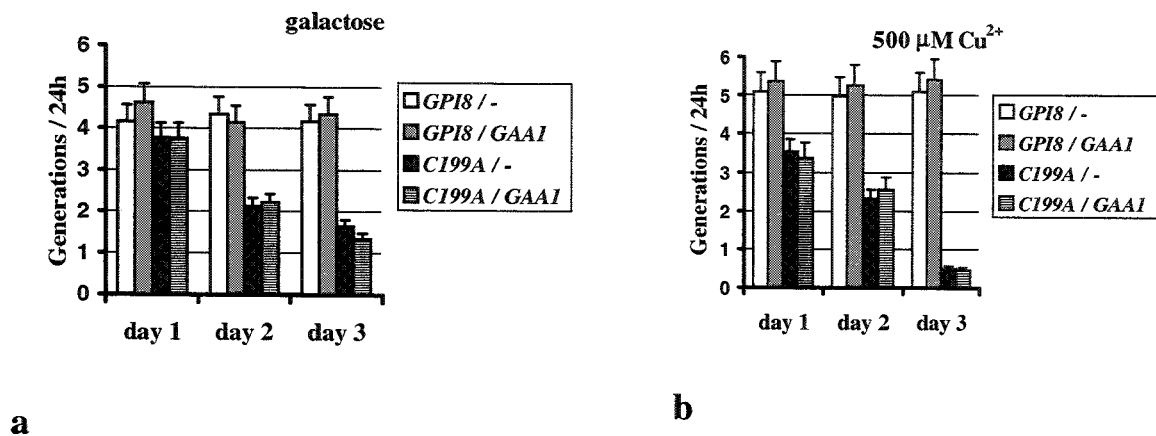
Overexpression of the dominant-negative active site mutant of Gpi8p, C199A, led to growth arrest of wt cells. This negative effect of C199A could be versed by co-overexpression of wt Gpi8p (Meyer *et al.*, 2000). Our hypothesis was that Gpi8p is a subunit of a complex and that mutant and functional Gpi8p molecules are in competition for entry into this complex which is essential for cell viability. The number of functional putative GPI:protein transamidase complexes might be determining cell viability. In order to increase the number of functional complexes under the condition where the C199A mutant is overexpressed, we envisaged that overexpressing another possible subunit of such a complex would lead to the formation of additional GPI:protein transamidase complexes, some with the C199A and others with the wt form of Gpi8p. If this were the case, two szenarios can be imagined: first, the cell dies even more rapidly than with only C199A overexpressed because there are too many non-functional complexes, or second, the cell starts to grow again since the cell has more functional complexes in absolute terms than with only C199A overexpressed. For example, assuming that a cell contains 20 molecules of genomically expressed wt Gpi8p and 200 copies of the overexpressed C199A mutant and only 11 molecules of a complex number-limiting subunit. Such a cell would contain 1 functional (wtGpi8p-subunit) and 10 non-functional complexes (C199A-subunit) if we assume that the affinity of both Gpi8p forms for the subunit are equal. Overexpression of the subunit 2-fold would lead to the formation of 2 functional (wtGpi8p-subunit) and 20 non-functional complexes (C199A-subunit); 20 non-functional complexes might kill a cell or 2 functional complexes might restore growth.

Since only scant information is available about the GPI:protein transamidase complex, it is very difficult to predict which scenario might be correct, or which other szenarios might occur. However, all these szenarios are highly speculative, and we decided simply to test this simple screen whether it might be appropriate to identify additional subunits of the GPI transamidase complex, since we had both the material and a functional plate assay already on hand.

#### 3.4.2. Results

We first wanted to know whether Gaa1p, another putative component of the GPI:protein transamidase might help a cell to overcome growth arrest caused by overexpression of the C199A mutant of Gpi8p. As it can be seen in Figure 1, overexpression of Gaa1p from multicopy vectors did not help to restore growth arrest of W303-1B wt cells overexpressing the active site mutant C199A. This might be explained in several ways, first another subunit but not Gaa1p is limiting for the number of complexes in a cell; second, Gaa1p together with another subunit is limiting for the number of complexes; third, overexpression of Gaa1p was not strong enough; fourth, our comprehension of the complex is wrong.

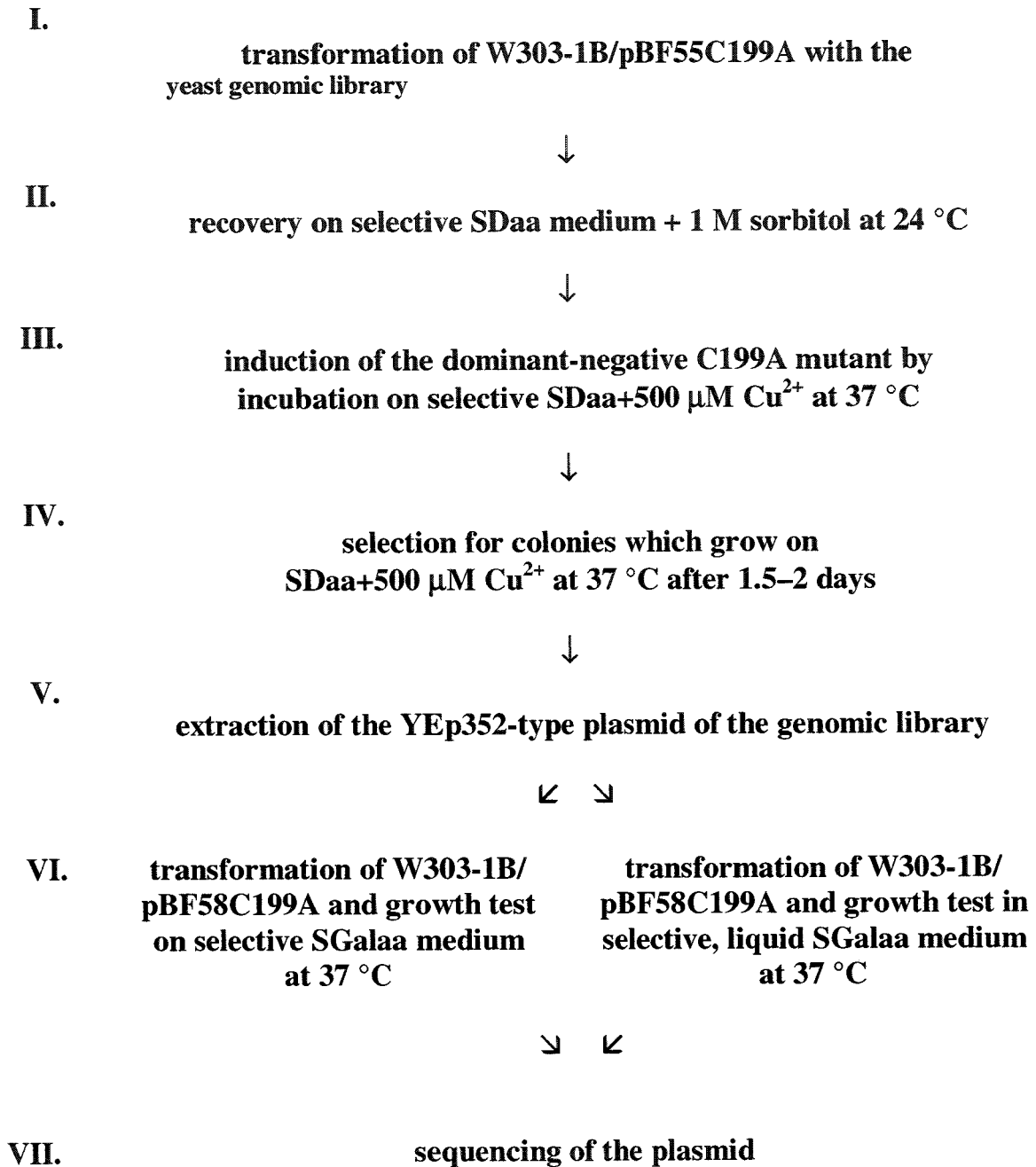
At least, overexpression of Gaa1p did not accelerate growth arrest by stabilizing non-functional Gpi8p (C199A). We therefore decided to perform a complementation analysis the Gpi8p-active site mutant induced growth arrest of wt cells by transformation with a genomic library. W303-1B wt cells were transformed with the multicopy plasmid pBF55C199A which harbors the active site allele C199A of *GPI8* under the control of the strong  $\text{Cu}^{2+}$ -inducible *CUP1* promoter. At a concentration of 300 to 500  $\mu\text{M}$   $\text{Cu}^{2+}$  on selective SDaa medium, these cells go through 2 or 3 cell generations and die, whereas the same cells containing in addition the multicopy vector YEp*GPI8* (Benghezal *et al.*, 1996) which expresses wt Gpi8p under the *GPI8* promoter, grow normally under the same conditions.



**Figure 1. The dominant negative effect of C199A cannot be suppressed or alleviated by co-overexpression of wt Gaa1p.**

Wt (*GPI8*) or C199A alleles of *GPI8* under the control of the galactose-inducible *GAL1-10* were chromosomally integrated using pBF57 and transformants growing on glucose were shifted to galactose (**panel a**). Alternatively, wt (*GPI8*) or C199A alleles of *GPI8* were expressed from a single copy vector under the control of the *CUP1* promoter (pBF54; **panel b**). In addition, the cells contained plasmids pDH17 and pDH15 (**panel a** and **panel b**, respectively) or the corresponding empty vectors (YEplac112 or YEplac195) for concomitant overexpression of *GAA1*. Exponential phase cells were cultivated at 24°C in selective glucose medium and expression of Gpi8p was induced by shifting to galactose containing medium (**panel a**) or by adding  $\text{CuSO}_4$  (**panel b**) and shifting cells to 37°C one hour later. Cell growth in quadruplicate cultures was monitored as described (Meyer *et al.*, 2000).

The conditions for the screen were optimized for a  $\text{Cu}^{2+}$  concentration of 500  $\mu\text{M}$  at which all C199A-expressing wt cells die without forming any false positive clones. A yeast genomic library containing genes under their natural promoter were overexpressed from a multicopy vector (YEp352) which is the same vector being used to produce YEp*GPI8* (Benghezal *et al.*, 1996). The screen was designed for multicopy suppressors of the growth arrest found in wt cell after induction of expression of the active site mutant C199A. The protocol of the screen is schematically represented in Figure 2.



**Figure 2. Protocol of the multicopy suppressor screen.**

W303-1B wt cells harboring the plasmid pBF55C199A were transformed with a yeast genomic library expressing yeast proteins under their natural promoter (step I) and recovered on 1 M sorbitol containing SDaa plates (step II). Overexpression of the active site mutant of Gpi8p, C199A, was induced by plating transformants onto selective SDaa plates containing 500 μM Cu<sup>2+</sup> (step III). Cells incubated at 37 °C grew for 2 to 3 generations and underwent growth arrest due to the dominant-negative effect of the C199A mutant. After 1.5 to 2 days, positive control cells (W303-1B/pBF55C199A/YEp*GPI8*) suppressing the dominant-negative effect of C199A by co-expressing wt *GPI8* from a multicopy vector under the control of the *GPI8* promoter were well distinguishable from negative control cells (W303-1B/pBF55C199A/YEp352) arresting growth because they only overexpress dominant-negative C199A and contain the empty vector YEp352 (step IV). YEp352-type plasmids stemming from the yeast genomic library were extracted from transformants which grew well after 2 days of incubation (step V), and were retransformed into wt cells containing the

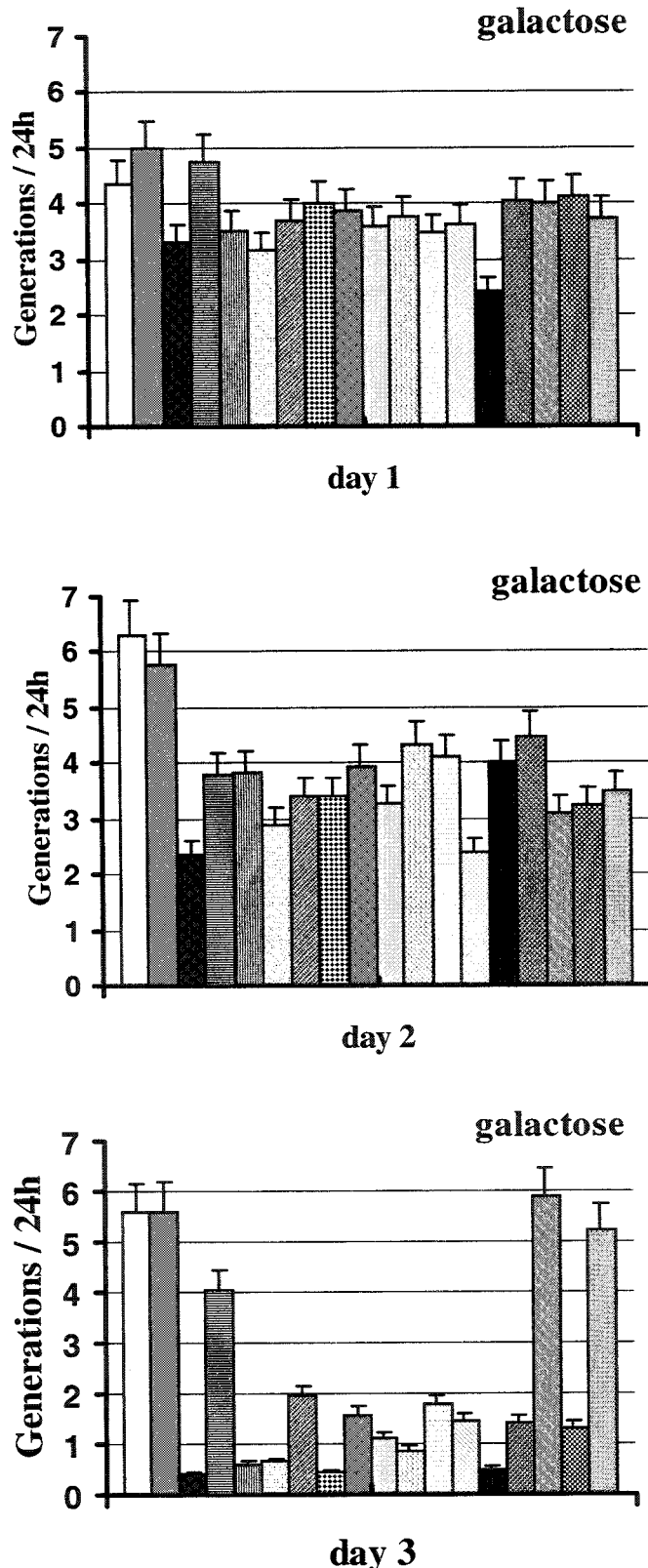
multicopy plasmid pBF58C199A which harbors the C199A mutant under the control of the galactose-inducible promoter *GAL1-10* promoter in order to reproduce the complementing effect of the library plasmid under  $\text{Cu}^{2+}$ -independent conditions (step VI, on the left). The same plasmids were retransformed into wt cells expressing the C199A mutant under the control of the *GAL1-10* promoter from a construct genomically integrated into the *LEU2* locus (step VI; on the right). These transformants allowed a more precise analysis of the complementing effect of the selected plasmids in liquid culture. Finally, plasmids showing the complementing effect under both the  $\text{Cu}^{2+}$ - and the galactose-dependent expression of the C199A mutant were sequenced to identify the ORFs being responsible for restoration of growth (step 7).

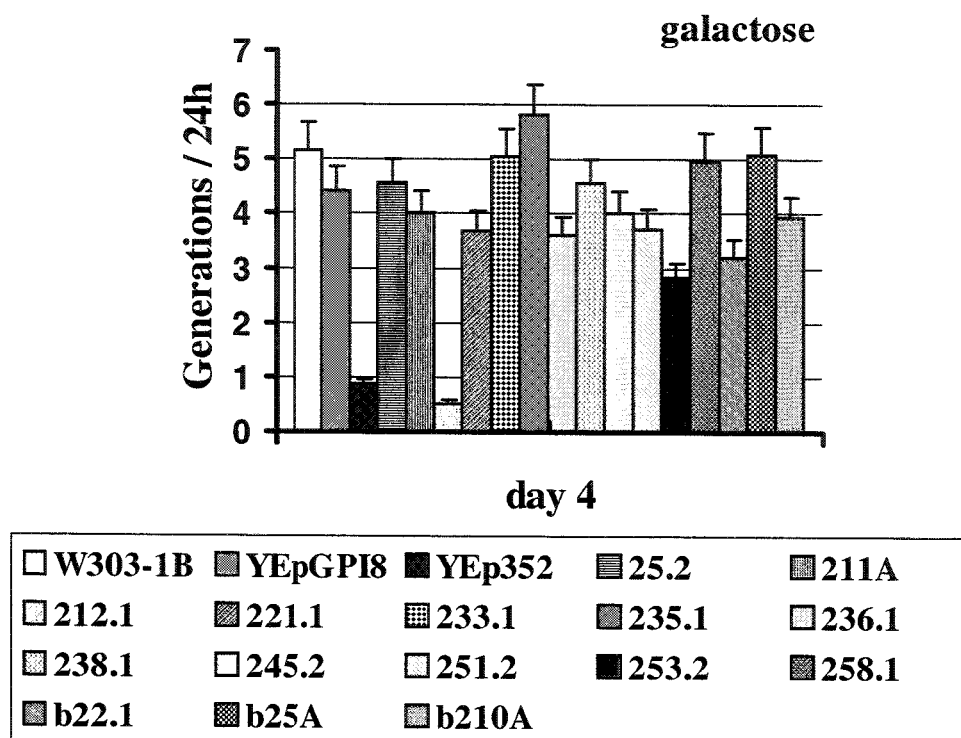
Altogether 23'500 clones were analyzed in step IV, an effort which led to the identification of 60 colonies which grew well after 2 days on 500  $\mu\text{M}$   $\text{Cu}^{2+}$ , and an additional 53 clones which showed an ambiguous phenotype. After 4 days of incubation about 1 to 2 % of the cells started to grow again as estimated from several plates with negative control cells. The complementing plasmid was then extracted from 11 out of the 60 well-growing clones and from 17 out of the 53 partially growing clones, retransformed into wt cells expressing the C199A mutant from a multicopy vector under the control of the *GAL1-10* promoter. 25 out of the 28 tested plasmids were also able to restore the C199A-affected growth in the galactose-dependent expression system (step VI, on the left). This indicated that the complementing effect of the isolated plasmids was not due to a reduction of the degree of overexpression of C199A by interference with the copper metabolism as for example would be expected for a plasmid overexpressing a putative  $\text{Cu}^{2+}$ -exporting protein. The same 28 clones were also replica-plated onto selective SDaa+5-FOA in order to force out the complementing plasmid, and were subjected again to a growth test in the presence of 500  $\mu\text{M}$   $\text{Cu}^{2+}$ . All but three showed again growth arrest indicating that the plasmid from the yeast genomic bank was responsible for the growth restoration in most of the positive clones. The three growing clones could be cells which either lost, reduced C199A expression or adapted itself to high C199A expression, however, they were not further analyzed as they were also the three clones which were not able to complement C199A-mediated growth arrest on galactose plates (step VI, on the left).

The plasmid shuffling on 5-FOA containing plates and the growth assay on galactose plates did not show a lot of false positives which indicates that the growth assay on  $\text{Cu}^{2+}$  was reliable. However, both tests did not help to distinguish between clones which show high and low complementing activity, and could therefore not serve as additional criterion to categorize complementing clones. Therefore, we decided to measure the growth in liquid culture which, we hoped would allow indirect quantification of the complementing activity of every single isolated plasmid and therefore, could act as a more restrictive criterion. We integrated the mutant allele C199A under the control of the *GAL1-10* promoter into the genomic *LEU2* locus in order to obtain a stable overexpression of the C199A mutant of approximately 10-12 times compared to wt level. These cells decelerate growth at about 24 h after induction of C199A expression, and are comparable to the situation depicted in Figure 1, panel A, but grow normally on galactose plates. For plate assays, C199A has to be expressed from a multicopy vector either under the control of the *GAL1-10* or the stronger *CUP1* promoter in order to achieve complete growth arrest of wt cells.

84 different plasmids from the original pool (60 well- and 53 partially growing clones) were retransformed into wt cells expressing the C199A mutant from the genomically integrated construct, and growth in liquid culture (galactose) was

measured over at least 4 days. Out of these 84 plasmids, 24 showed strong complementing activity. The best candidate for such a complementing gene would be *GPI8* itself. Therefore, all 24 plasmids were subjected to restriction analysis which should reveal the presence of the *GPI8* gene. Digestion with the restriction enzymes BsmI and BstXI should reveal a typical band of 613 bp which was found for 9 of the 24 plasmids. For two of them, the presence of the *GPI8* gene was confirmed by sequencing. All the other 15 clones which showed a restriction map lacking the 613 bp band were sequenced (Table 1) and subjected to further analysis. First, a precise growth curve over 4 days was measured (Figure 3).





**Figure 3. Growth curve of multicopy suppressors in presence of C199A.**

The C199A allele of *GPI8* under the control of the galactose-inducible *GAL1-10* was chromosomally integrated into W303-1B by using pBF59C199A, and transformed with the 15 complementing multicopy plasmids from the yeast genomic library, YEp*GPI8* (positive control) or YEp352 (negative control). Furthermore, untransformed W303-1B was analyzed side-by-side with the transformants. Cells were cultivated at 24°C in selective glucose medium and expression of mutant Gpi8p was induced by shifting to galactose containing medium and shifting cells to 37°C one hour later. Cell growth in quadruplicate cultures was monitored over a period of 4 days as described (Meyer *et al.*, 2000). The graph reflects growth at day 4 after the shift to galactose.

Complementing plasmids can be categorized into two classes: those which suppress the C199A-induced growth arrest already from the beginning (permanent suppressors) and those which efficiently suppress only during the fourth day (late suppressors). Permanent suppressors grow well during the whole analysis and behave like the positive control (YEp*GPI8*). In the negative control (YEp352), wt cells generally show significant growth during the first 2 days after the shift from glucose to galactose, and growth arrest is well detectable always at the beginning of the third day. Permanent suppressors are contained on plasmids 25.2, b22, and b210A. Plasmid 212.1 did not show any suppressive effect although it passed all the other tests, therefore it is probably worthwhile to repeat its analysis. All the other plasmids contained late suppressors.

Sequencing of the suppressor plasmids revealed several ways a cell might cope with the overproduction of a dominant-negative *gpi8* allele (Table 1). Several genes might be involved in down-regulation or degradation of mutant Gpi8p, as for example Rub1 which is an ubiquitin-related protein which might be involved in degradation, or RNH1, a gene encoding for the ribonuclease H, Ath1, a vacuolar acid trehalase, Gim4 which is a chaperone, Ynl313c having a tetratricopeptide repeat which was found -

among other proteins - also in transcription repressors, and *Rim13*, a calpain-like protease. Sec18p and transposon sequences, the later containing the gene for reverse transcriptase, might also be counted to the same subset of isolated genes, however, ideas about their role in reducing the dominant-negative effect of C199A overexpression remain speculative. Overexpressed Sec18p might improve the efficiency of vesicular traffic and thus be responsible for a more effective regeneration of the ER.

**Table 1. Sequences of permanent and late suppressor plasmids.**

plasmid	sequence <sup>†</sup>	complete gene	description of the complete gene <sup>Φ</sup>	N-ter. SS <sup>#</sup>	TMD #	deletant
25.2	YLR015w-	YLR016c	u., homology to u. <i>C. elegans</i> protein;	no	0	viable
	YLR016c-MEU1		Meu1p: regulates ADH2 expression	no	0	viable
			Ylr015w ( <i>BRE2</i> ): mutant is sensitive to Brefeldin A	no	0	viable
b22	HPR1-RUB1-	RUB1	ubiquitin-related protein;	no	0	viable
	YDR140w		Ydr140w: motif of S-adenosylmethionine-dependent methyltransferases	no	0	viable
			Hpr1p: maintaining stability of direct repeat sequences	no	0	viable
b210A	YCL075w- YCL074w- YCLWΩ2	YCL074w	reverse transcriptase	n.d.	n.d.	u.
211A	ribosomal RNA- URA3	none	Ura3p: orotidine-5'-phosphate decarboxylase	no	0	viable
212.1	RNH1	none	Rnh1p: ribonuclease H, endonuclease, degrades RNA in RNA/DNA hybrids	no	0	viable
221.1	BOP3-YNL041c	BOP3	u., Bypass Of PAM1;	no	0	viable
			Ynl041c: u.	no	0	u.
233.1	ENA1	none	Ena1p: P-type ATPase involved in Na <sup>+</sup> and Li <sup>+</sup> efflux	no	9	viable
235.1	RIM13-	none	Rim13: protease	no	0	viable
	YMR155w		Ymr155w: u.	no	9	viable
236.1	YML066c-ORC1	none	evtl. u. gene between the 2 genes;	n.d.	n.d.	u.
			YML066c: u.	cleavable	4	u.



238.1	DAL82-	YNL313c	tetratricopeptide repeat; homolog in <i>C.elegans</i> ;	no	0	lethal
	YNL313c-RFA2		Rfa2p: DNA replication factor A, 36 K subunit	no	0	lethal
			Dal82p: transcriptional activator for allantoin catabolic genes	no	0	viable
245.2	Ty-1 short transposon sequence					
251.2	Ty-1 transposon					
253.2	not sequenced					
258.1	PCA1	none	Pca1p: putative P-type Cu <sup>2+</sup> -transporting ATPase	no	8	viable
b25A	RPG1-SEC18-	SEC18	yeast NSF, ATPase required for vesicle fusion;	no	0	lethal
	SPT7		Spt7p: component of nucleosomal histone acetyltransferase complex	no	0	viable
			Rpg1p: translation initiation factor eIF3, p110 subunit	no	0	lethal
11	YEA4-GIM4-	GIM4	prefoldin, chaperone ( $\alpha$ , $\gamma$ -tubulin);	no	1	viable
	WBP1		Wbp1p: $\beta$ subunit of OTase <sup>Ψ</sup> ;	cleavable	1	lethal
			Yea4p: similar to <i>K. lactis</i> UDP-GlcNAc transporter	no	6	viable
29	ATH1	none	Ath1p: vacuolar acid trehalase	no	1	viable

II The approximate DNA sequence present on the sequenced plasmids is indicated by borders.

Φ For plasmids which do not contain a complete gene, the indicated gene fragment is described in the following. For plasmids which contain, in addition to the complete gene, gene fragments, the latter are described as indicated.

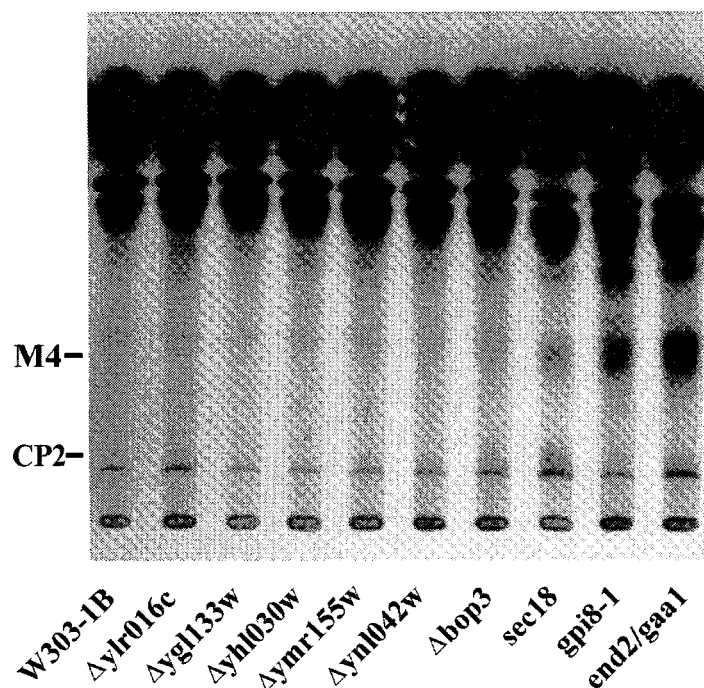
# The presence of an N terminal signal (N-ter. SS) sequence and the number of transmembrane domains (TMD) as determined with the PSORTII software

Ψ oligosaccharyl transferase complex for N glycosylation

n.d.: not determined; u.: unknown

However, several unknown genes were isolated for which a function is difficult to predict from BLAST searches. *YLR016c*, *BOP3*, *YNL041c* and the putative gene on plasmid 236.1 located between the ORFs of *YML066c* and *ORC1* which might not have been detected by the ORF prediction tools in SGD and YPD. However, it is not clear whether *YNL041c* is expressed, since only a 5' end fragment of the complete gene sequence is present on the plasmid. All plasmids contain in addition to the

complete gene sequence gene fragments of which it is unknown whether they play a role in the suppressive effect. Plasmids 258.1, 233.1 and 235.1 for example contain fragments of genes encoding for putative transmembrane transporters; Pca1 is a putative P-type  $\text{Cu}^{2+}$ -transporting ATPase, if its gene fragment is functional, its complementing activity would be easy to explain in the  $\text{Cu}^{2+}$ -dependent expression of C199A but not in the galactose-dependent analysis described in Figure 3; Ena1p is a P-type ATPase involved in  $\text{Na}^+$  and  $\text{Li}^+$  efflux, and Ymr155w is an unknown member of the MFS (multifacilitator superfamily) transporter superfamily. A precise localization of the suppressive activity has to be performed by growth analysis with plasmids each containing only a single gene.



**Figure 4. Viable deletants do not accumulate CP2.**

Analyzed strains include deletants of ORFs isolated in this multicopy suppressor screen, or in the case of *sec18*, a temperature-sensitive mutant allele. *Δygl133w* is an ORF which did not pass step VI of the screen but had been sequenced before. *Δyhl030w* is identical with *Δecm29* and was isolated in the remodelase screen (chapter 3.6.1.). *Δynl042w* is identical with *Δbop3* but was obtained directly from EUROSCARF. Cells were grown to exponential phase in SDaa medium at 24 °C. After a preincubation of 45' at 37 °C, cells were resuspended in fresh SDaa medium at 10 OD/ml, and preincubated at 37 °C was prolonged for another 15'. Cells were labeled with [2-<sup>3</sup>H]-*myo*-inositol for 20', diluted with prewarmed fresh SDaa medium and incubation was prolonged for 35'. Lipids were extracted, desalted, and separated by TLC. Radioactivity was visualized by fluorography.

In a first analysis deletant strains of some of the unknown clones were labeled with [2-<sup>3</sup>H]-*myo*-inositol at 37 °C in order to explore the lipid profile for the accumulation of complete GPI precursor glycolipids. As can be seen in Figure 4., all deletant strains showed a wt lipid profile and did not accumulate any lipids. However, it might be worthwhile to repeat the analysis since the accumulation of CP2 by *gpi8-1* was not well detectable even when the TLC was overexposed. Nonetheless, *gpi8-1* showed a

strong accumulation of M4. Moreover, none of these deletants revealed accumulation of immature forms of the yeast GPI proteins Cwp1 or Yap3p as assessed by immunoblots (data not shown). Altogether, this indicates that the ORFs analyzed so far are either not involved in GPI anchor addition or play a role in GPI anchor addition which is redundant, i.e. can be also be exerted by another gene. At all events, their loss does not lead to a detectable defect in GPI anchor addition as analyzed by the accumulation of both substrates of the putative GPI:protein transamidase. Analysis of the remaining ORFs will enable a final assessment of the results of this multicopy suppressor screen.

### 3.4.3. Discussion

A multicopy suppressor screen was performed by selecting for yeast genomic genes which help to overcome growth arrest of wt cells caused by overexpression of C199A, an active site mutant of Gpi8p. This screen was a trial to identify other proteins required for GPI anchor attachment. Considering the concept of the screen several other genes might have a suppressive activity:

First, the *GPI8* gene; this gene is expected to complement best the C199A-induced growth arrest. Indeed more than one third (9) of the best growing transformants (step VI on the right) contained a plasmid with the *GPI8* gene.

Second, the *TRP1* gene; this gene might have helped to force out the *TRP1*- based pBF55C199A or pBF58C199A (step III and step VI on the left). Indeed, a few plasmids displaying only a partial suppressive effect were sequenced as well, and one of them contained *TRP1*.

Third, genes which are involved in protein degradation which might accelerate degradation of the dominant-negative C199A mutant when overexpressed. Rub1p, a ubiquitin-related protein, might be such a candidate.

Fourth, genes which block or impair expression of the C199A mutant on the RNA level. This screen identified f.ex. the ribonuclease H. Ynl313p has a tetratricopeptide repeat which was observed in transcription repressors. Probably also reverse transcriptase of Ty-1 might work in this way. However, all these explanations are highly speculative.

Fifth, genes involved in plasmid maintenance; genes involved in such a mechanism could account for lowered overexpression of C199A by partial plasmid loss or downregulation of the copy number. No such gene was identified eventually because these genes were lost during step VI (on the right) since there C199A was expressed from a chromosomally integrated construct.

Sixth, a gene encoding an essential GPI protein, such as for example Rot1p; by overexpressing GPI anchor attachment would be favored compared to the other normally expressed GPI proteins. However, it is not probable that cell growth could be restored by the overexpression of only 1 essential GPI protein, since other GPI proteins might also be essential and a total lack of all other non-essential GPI proteins would probably also lead to cell death since for example growth of *Δgas1* cells is already highly impaired.

Although the analysis of the screen isolates is not yet accomplished, several unknown genes were identified which most probably belong to one of the 7 gene classes mentioned above: *YLR016c*, the gene of plasmid 236.1, *YNL313c*, *BOP3*, *YMR155w*;

among the few plasmids with a low complementing activity which were sequenced was established: *YGL133w* and *YPL052w*, both proteins being unknown and not predicted to contain a transmembrane domain. Whether one of them is involved in GPI anchor addition remains to be elucidated.

Our screen selected for “best growing clones” (step VI, on the right). It may be worthwhile to sequence all the other plasmids which show a weak complementing activity. Obviously, they may express genes having a pleiotropic effect and help a cell to partially overcome C199A-induced growth arrest; however, in the best case, they also might overexpress a subunit which helps partially to increase the number of functional GPI:protein transamidase complexes.

#### 3.4.4. Experimental Procedures

General information on strains, plasmids and protocols for the cultivation of yeast cells can be found in chapter 5. Plasmids were sequenced with the primers M13 and M13r at Microsynth (Balgach). Growth curves and radiolabeling of GPI lipids were performed as described (Meyer *et al.*, 2000; chapter 3.1).

#### 3.4.5. Acknowledgements

We would like to thank Dr Howard Riezman for the generous gift of Gaalp-expressing plasmids, Dr Markus Aebi for the yeast genomic library, and the EUROFAN I members for providing us with yeast knock out strains. We are greatly indebted to Carole Roubaty for excellent technical assistance.

### 3.5. Isolation of *gpi8* mutants displaying a temperature sensitive growth phenotype

#### 3.5.1. Introduction

For a synthetic lethality screen the use of a mutant having a strong conditional defect such as a temperature-sensitive growth defect is required. Such a strong defect increases the chance that another gene in the same cell if mutated becomes synthetic lethal to the known mutant. The mutant *gpi8-1* is temperature-sensitive for the accumulation of the complete GPI precursor CP2 and of the immature ER form of the GPI protein Gas1p. However, its temperature-sensitive phenotype for growth is weak. At the restrictive *gpi8-1* cells express a reduced amount of wt Gpi8p in the range of 50% compared to W303-1B wt cells which leads to a lengthening of the cell cycle by 5 to 20 % compared to wt cells. Therefore, *gpi8-1* is not appropriate to screen for additional genes involved in GPI anchor addition by a synthetic lethality screen.

Therefore we decided to produce a *gpi8* mutant showing a strong temperature-sensitive growth phenotype. Such a mutant should grow well at 24 °C, but not at 37 °C, i.e. the mutant phenotype should be manifested rapidly at 37 °C and should be stable. A screen for synthetic lethality with a known mutant is suggested to be more appropriate for the discovery of additional genes involved in GPI anchor attachment than the “non-classical” multicopy suppressor screen as described in chapter 3.4. since most of the novel genes which are isolated belong or interact directly with the same metabolic process as the mutant and the range of false positives should be much more limited. (A classical multicopy suppressor screen selects for genes which, when overexpressed, complement or alleviate the phenotype of a mutated genomic gene being generally not overexpressed. In a classical multicopy suppressor screen, genes reducing protein expression, which were picked up frequently in our previous screen, would in general not get through.). However, the disadvantage of a synthetic lethality screen compared to a multicopy suppressor screen is that cloning of the novel gene is often difficult and time-consuming.

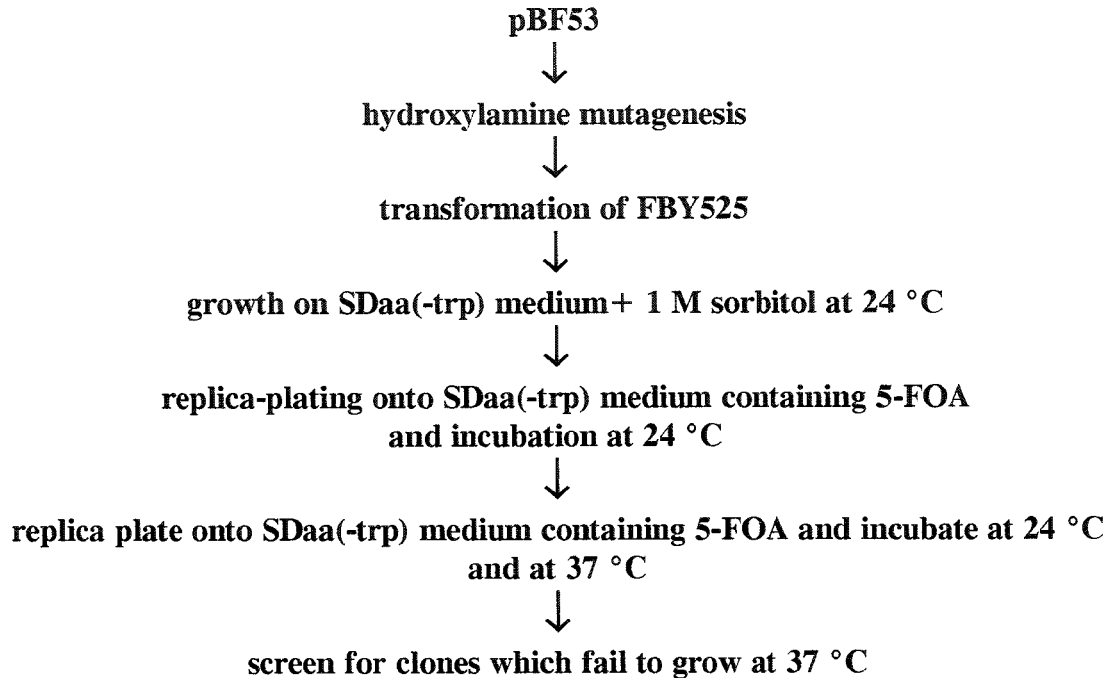
#### 3.5.2. Results

##### *Screen for ts gpi8 mutants by hydroxylamine mutagenesis and plasmid shuffling*

The screen to isolate temperature-sensitive (ts) alleles of the *GPI8* gene was performed as basically described for the isolation of the temperature-sensitive *dpm1* mutant (Orlean, 1990).

The *GPI8* gene on the single copy vector pBF53 was mutagenized *in vitro* with hydroxylamine as described (Rose and Fink, 1987) and used to transform strain FBY525. The chromosomal copy of the essential *GPI8* gene in this strain is disrupted ( $\Delta gpi8$ ; Benghezal *et al.*, 1996), but the strain is kept alive because it harbors the multicopy plasmid YEp*GPI8* (Benghezal *et al.*, 1996) bearing the wt allele of the *GPI8* gene. After transformation of strain FBY525 with the mutagenized pBF53\* plasmid, the plasmid shuffling technique (Boeke *et al.*, 1987) was used to force out the plasmid YEp*GPI8* which contains the selective marker gene *URA3*, and in this way to isolate transformants that relied only on the mutagenized plasmid for growth at

a permissive temperature of 24 °C but failed to grow at 37 °C. The protocol for the plate assay is summarized in Figure 1.



**Figure 1. Procedure to isolate temperature-sensitive *gpi8* alleles.**

The single copy vector pBF53 (*TRP1*, *GPI8*) was mutagenized by incubation with hydroxylamine. DNA was precipitated and used for transformation of strain FBY525 ( $\Delta gpi8/YEpGPI8$ ). Recovered cells were replica-plated onto 5-FOA to force out the plasmid YEp*GPI8* and growing cells were then subjected to a comparative growth test at 24 and 37 °C to isolate temperature sensitive colonies.

About 300 of totally 12'000 analyzed colonies showed a temperature-sensitive phenotype for growth on SDaa(-trp) medium and were further analyzed to separate ts *gpi8* alleles from plasmid-unlinked mutants, temperature-sensitive *trp1* mutants and non-conditional *gpi8* mutants. Temperature-sensitive mutants not related to the plasmid and temperature-sensitive *trp1* mutants were identified and discarded by selecting for FBY525 strains harboring both the mutagenized pBF53\* and YEp*GPI8* plasmids which didn't grow at 37 °C on YPD plates and SDaa(-trp) plates, respectively. Ts *gpi8* mutants should grow well at any temperature under these conditions. Non-conditional mutants were separated from ts *gpi8* mutants by analyzing growth of transformants after incubation on 5-FOA, on SDaa(-trp) at 24 and 37 °C. Non-conditional mutants grow badly at both temperatures whereas ts *gpi8* mutants grew well at 24 and died at 37 °C. The 300 tested strains were reduced to a number of 41, of which the best 7 were analyzed in more detail. Growth at 24 and 37 °C was analyzed on plates and in liquid medium (Table 1).

**Table 1. Ts phenotype for growth of *gpi8* alleles on solid and in liquid medium.**

	1 colony was resuspended in 1 ml H <sub>2</sub> O; 2 µl of this suspension was spotted on solid SDCUA, incubated for 1 night at 24 °C and then 1 plate was shifted to 37 °C (results after 1.5-2.5 d)			1 colony was grown up in SDCUA to exponential phase (1.5-2 d); cells were diluted and plated out on SDCUA (≈ 200 cells/plate) (results after 1.5-2.5 d)			Cells were cultured to exponential phase in SDCUA (1½ d); diluted in SDCUA, and split; growth was analyzed at 24 and 37 °C (growth after 8 h)	
	24 °C	37 °C	rev.	24 °C	37 °C	rev.	24 °C	37 °C
<b>W303-1B</b>	+++	+++		+++	+++		+++	+++
<b>FBY526</b>	++(+)	+++		++(+)	+++		++(+)	+++
<b><i>gpi8-1</i></b>	+++	++		+++	++		+++	++(+)
<b><i>sec18</i></b>	+++	-	-	+++	-	-	+++	-
<b>clone 5</b>	++(+)	+	+	++(+)	+	-	++(+)	++
<b>clone 6</b>	++(+)	-	+	++(+)	+	-	++(+)	++
<b>clone 18</b>	++(+)	+	-	++(+)	++	-	++(+)	++
<b>clone 20</b>	++(+)	++	+	++(+)	++	-	++(+)	+++
<b>clone 23</b>	++(+)	+	-	++(+)	++	-	++(+)	++
<b>clone 34</b>	++	-	-	++	-	10 %	++	+
<b>clone 41</b>	++(+)	+	-	++(+)	+	-	++(+)	++

FBY526 cells (positive control) are  $\Delta gpi8$  cells stemming from FBY525 which harbor unmutagenized pBF53 instead of YEp*GPI8*.

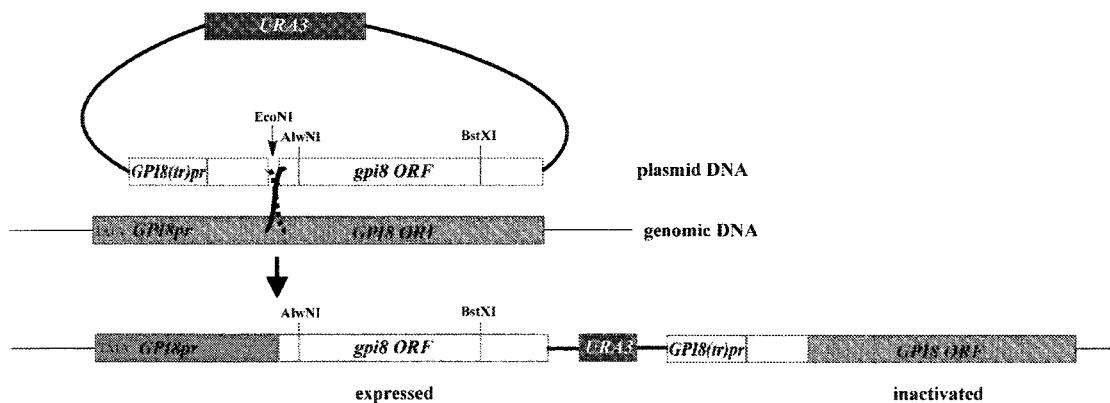
As it can be seen in Table 1, certain of the analyzed clones formed revertants. These revertants might be cells which eventually could overcome the temperature-shift by increasing the number of the single copy vector pBF53\* bearing the mutagenized *gpi8* allele, and in this way, compensate the reduced Gpi8p activity. This problem could maybe be solved by integrating *gpi8* alleles into the genome of  $\Delta gpi8$  cells. But before that, additional growth tests were performed which are summarized in the following: plate tests over 6-8 days revealed that the ts phenotype for growth of non-reverting clones are: 5 > 18 > 23 >> 20, 41. Clone 6 grew very well at 24 °C, still a little bit at 37 °C, and formed reproducibly revertants after 6 to 8 days. Clone 34 grew badly already at 24 °C, failed to grow at 37 °C and formed revertants in an unpredictable manner. Clone 5 still grew at 37 °C and has been shown to form revertants in only a single experiment (1 out of 6).

All 7 clones were then analyzed for accumulation of the complete GPI precursor glycolipid CP2 and of the immature GPI protein precursor of Gas1p. Cells were labeled with [2-<sup>3</sup>H]-*myo*-inositol, lipids extracted, desalted and separated by TLC. Detection by fluorography revealed that only clone 18 very weakly accumulated CP2, whereas all the other isolated clones showed a wt lipid profile. Furthermore, none of the clones accumulated the immature 105 kD form of Gas1p significantly (data not shown). All results taken together let us come to the conclusion that our mutants - although showing a temperature-sensitive phenotype which was stronger than that of *gpi8-1* - were still not appropriate for a synthetic lethality screen. The partially unpredictable appearance of revertants and the absence of a GPI anchoring defect forced us to undertake another selection procedure for temperature-sensitive *gpi8* alleles.

**Screen for *ts* *gpi8* mutants by integration of random PCR mutagenized *gpi8* alleles into the genomic *GPI8* locus of wt cells**

Another screen for temperature-sensitive *gpi8* mutants was performed by exploiting *gpi8* alleles mutagenized by random PCR (cf. chapter 3.2.). These *gpi8* alleles contain mutations in the ER luminal part of the *GPI8* gene corresponding to the 1074 bp AlwNI/BstXI fragment of the *GPI8* ORF (amino acid positions 26 to 385); the sequence for the N terminal signal sequence and the C terminal part including the transmembrane domain and the short cytosolic stretch show wt sequence (Figure 2).

W303-1B wt cells were transformed with the yeast integrative plasmid library pBF515\* bearing a non-functional truncated *GPI8* promoter fused to *gpi8* alleles mutagenized by random PCR mutagenesis. Furthermore, these constructs contain an EcoNI silent site within the N terminal signal sequence at position +54 (amino acid position 18) which allows site-directed integration into the genomic *GPI8* gene. The integration event occurs by homologous recombination and should lead to inactivation of the the genomic *GPI8* ORF which is fused by the integration event to the non-functional truncated *GPI8* promoter previously present on the integrative plasmid. At the same time, the mutagenized *gpi8* allele is fused to the functional wt *GPI8* promoter of the endogenous *GPI8* gene and becomes expressed as the only *GPI8* allele (Figure 2).



**Figure 2. Substitution of expressed *GPI8* alleles by homologous recombination.**

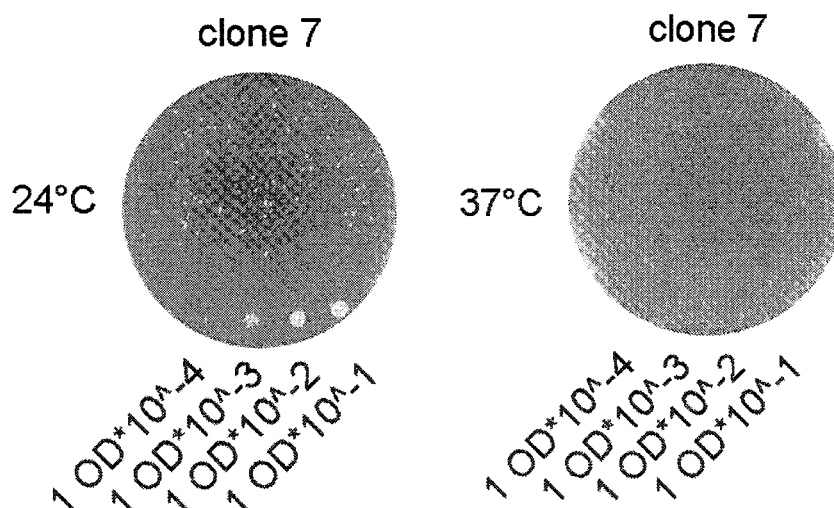
W303-1B wt cells were transformed with a *gpi8* allele library on the integrative plasmid pBF515 (*URA3*). The wt *GPI8* promoter on this plasmid (*GPI8(tr)pr*) is truncated and lacks the TATA box which renders it non-functional. The *gpi8* alleles present on pBF515 contain in addition an *EcoNI* cleavage site and a mutated AlwNI/BstXI fragment which was mutagenized by random PCR and introduced from the *gpi8* allele library YEp*GPI8*. For directing integration, pBF515 was linearized with *EcoNI* prior to electroporation of wt cells. The integration event occurs by homologous recombination and leads to an inactivation of the endogenous *GPI8* and expression of the mutated *gpi8* allele from the plasmid. Transformants are then tested for growth on SDaa medium at 24 and 37 °C.

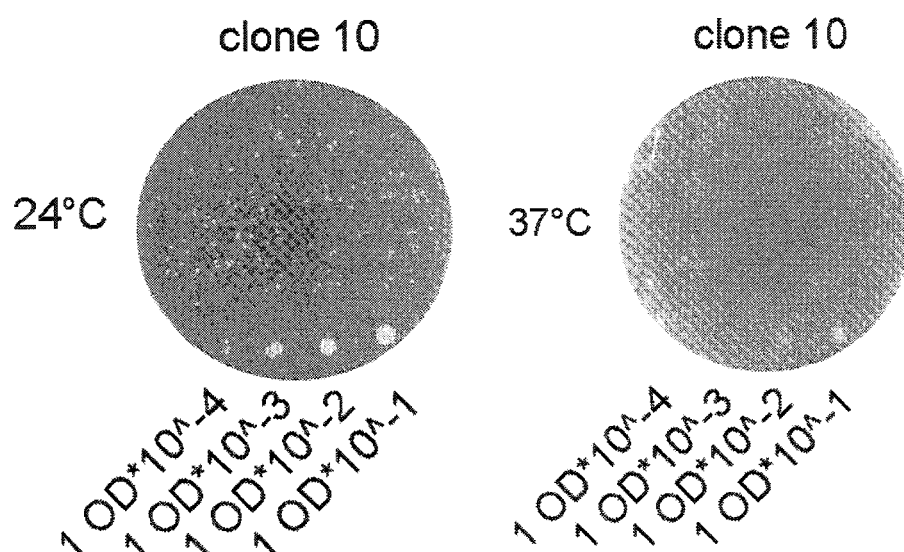
Two control plasmids were constructed to test whether the integration event as depicted in Figure 2 might be successful. First, a construct with the truncated *GPI8*



promoter of plasmid pBF515 fused to the wt *GPI8* gene was ligated into the single copy vector YCplac22 resulting in the pBF53-type plasmid pBF521. pBF521 was transformed into FBY525 ( $\Delta gpi8/YEpGPI8$ ), and transformants were transferred onto 5-FOA. None of the transformants showed growth by this plasmid shuffling analysis indicating that the promoter was non-functional (data not shown). Second, substitution of endogenous *GPI8* expression by expression of the transformed *GPI8* alleles was tested with the plasmid pBF522 which is a pBF515-type plasmid containing the wt *GPI8* fused to a C terminal myc-his6 tag. Transformation of W303-1B cells resulted in expression of the myc-his6 Gpi8p only as assessed from immunoblots (data not shown). This indicates that the integration event was correctly directed and led to an inactivation of the endogenous *GPI8* ORF and expression of the *GPI8* allele stemming from the integrative plasmid.

After transformation with pBF515 containing the *gpi8* allele library, wt cells were recovered on selective SDaa+1 M sorbitol medium, and transformants were replica-plated onto SDaa medium and incubated at 24 and 37 °C. Colonies which grew well at 24 °C but not at 37 °C were selected as putative temperature-sensitive *gpi8* mutants. Approximately 10'200 transformants were subjected to growth analysis which lead to the isolation of about 20 colonies which showed impaired growth at 37 °C. All this 20 clones were analyzed in a second round again for a strong temperature-sensitive growth phenotype. Out of the 20 analyzed clones, 3 displayed the desired features: growth comparable with wt cells at 24 °C, and a rapid and complete growth arrest at 37 °C (Figure 3). As clones 7 and 10, clone 3.1 showed a strong ts phenotype for growth, whereas wt cells grew happily at both 24 and 37 °C (data not shown).

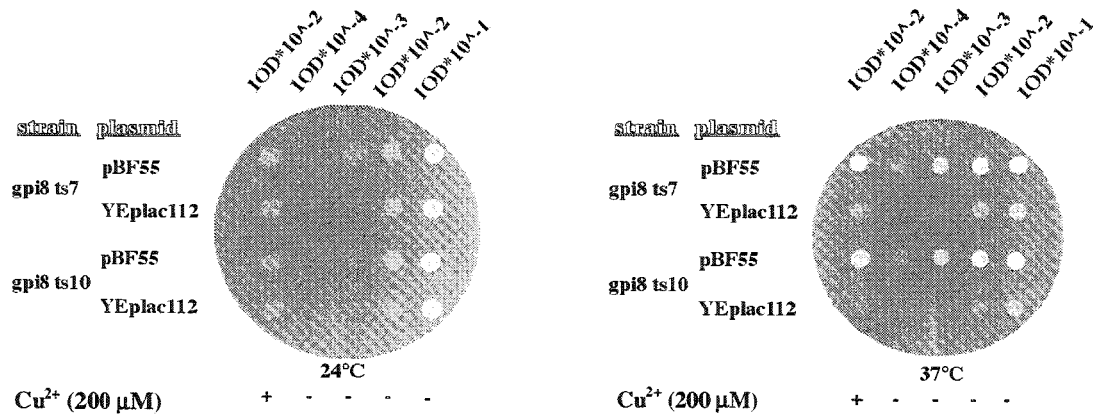




**Figure 3. Clone 7 and 10 are temperature-sensitive for growth.**

Fresh liquid cultures were diluted to  $10^{-4}$  or as indicated and plated or spotted onto selective SDaa medium. After 3 days of incubation at 24 and 37 °C, growth was assessed.

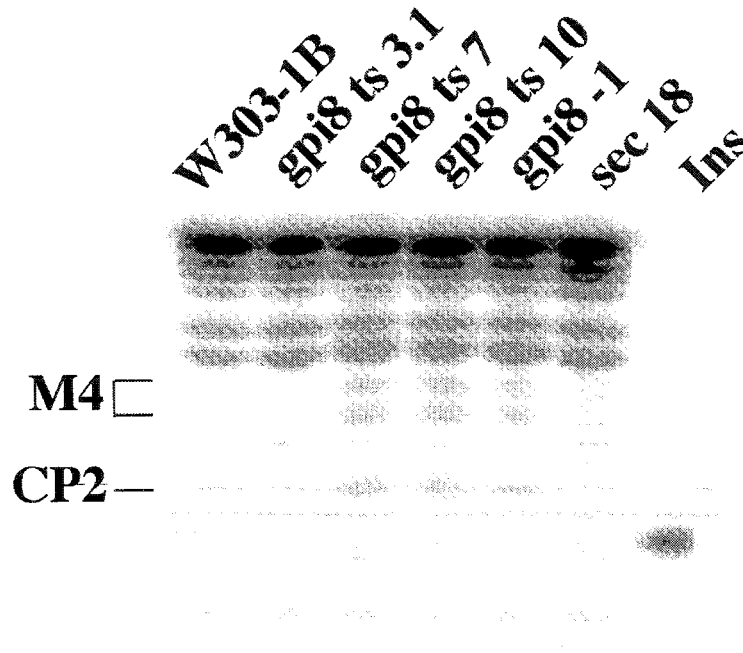
Furthermore, it was tested whether the ts phenotype of the isolated temperature-sensitive mutants was due to a mutation in the *GPI8* allele and could be complemented by overexpression of wt *GPI8*. Therefore, all three clones were transformed with the multicopy plasmid pBF55 which harbors the wt *GPI8* gene under the control of the strong  $\text{Cu}^{2+}$ -inducible *CUP1* promoter or the empty vector YEplac112. The *CUP1* promoter is not tight and expression of Gpi8p amounted to about 80-200 % of wt level depending on the medium already when no copper was added. As can be seen in Figure 4, overexpression of wt *GPI8* led to a restoration of growth at 37 °C in clones 7 and 10 indicating that the temperature-sensitive phenotype for growth was due to impaired Gpi8p function. Clone 3.1 was also transformed with the same plasmids and growth of transformants was analyzed at the permissive and restrictive temperature in liquid culture. Even strong overexpression of wt *GPI8* (at  $\text{Cu}^{2+}$  concentrations of 200 and 500  $\mu\text{M}$ ) could not restore growth at 37 °C (data not shown). This indicates that the severe growth defect at 37 °C is not linked to the mutation of the expressed *gpi8* allele or that the expressed *gpi8* allele is a very strong dominant-negative mutant.



**Figure 4. Complementation of the ts growth phenotype of mutants 7 and 9 by overexpression of wt *GPI8***

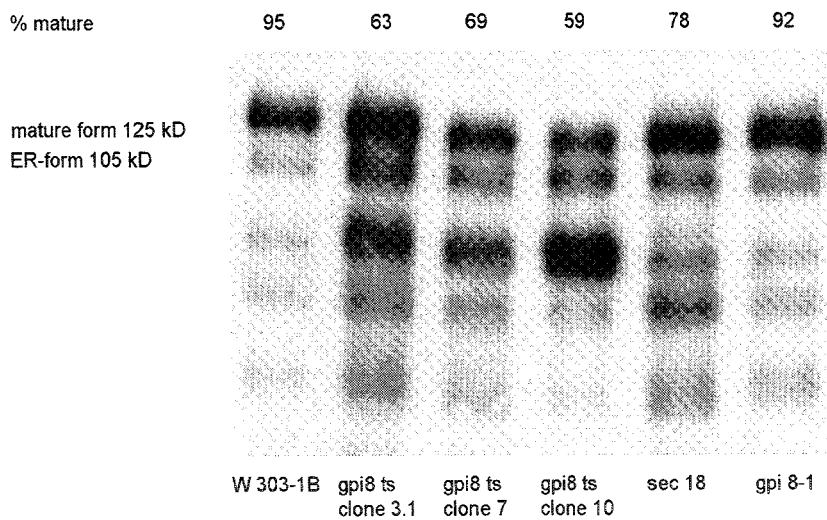
Both mutants were transformed with the multicopy plasmid pBF55 harboring the wt *GPI8* gene under the control of the *CUP1* promoter and empty vector YEplac112 by the LiAc method. Exponential phase cells were diluted as follows:  $1 \text{ OD/ml} \times 10^{-1}$ ,  $1 \text{ OD/ml} \times 10^{-2}$ ,  $1 \text{ OD/ml} \times 10^{-3}$ ,  $1 \text{ OD/ml} \times 10^{-4}$ . 5  $\mu\text{l}$  of each dilution were spotted onto plates. The first line contains spots of a  $1 \text{ OD/ml} \times 10^{-2}$  dilution supplemented with 200  $\mu\text{M}$  of  $\text{Cu}^{2+}$ . The complementing effect of *GPI8* overexpression is already visible after an overnight incubation, and was confirmed by a prolonged incubation of 2 days (not shown).

Moreover, all three mutants were tested for accumulation of the complete GPI precursor glycolipid CP2 and the immature ER form of Gas1p as further criteria for impaired *GPI8* function. *gpi8* ts mutants 7 and 10 strongly accumulated CP2, whereas the mutant 3.1 didn't show any accumulation (Figure 5). However, the mutant 3.1 heavily accumulated the immature 105 kD form of Gas1p supposing that this mutant might also be impaired in *GPI8* function although it does not show any accumulation of CP2 and its temperature-sensitive growth defect could not be complemented by overexpression of wt *GPI8*. Further analysis of this mutant including sequencing should reveal whether an expected mutation in the expressed *gpi8* allele is present and accounts for accumulation of Gas1p.



**Figure 5. Accumulation of CP2 in *gpi8* ts mutants.**

Exponentially growing W303-1B, FBY11 (*gpi8-1*), and *sec 18* cells together with the three temperature-sensitive mutants 3.1, 7 and 10 were shifted from 24 to 37 °C, preincubated for 20 ', labeled with [2-<sup>3</sup>H]-myo inositol at 37 °C for additional 20 min, diluted with medium and incubated for further 40 min. After addition of NaN<sub>3</sub>/NaF (10 mM of each), the lipids were extracted, purified and run on an analytical TLC by using the solvent system chloroform/methanol/water (CMW) 10:10:3. Detection of radiolabeled lipids was performed with a Berthold scanner.



**Figure 6. Accumulation of immature Gas1p at 37 °C.**

Exponential phase cells were shifted from 24 to 37 °C, and preincubated for 1 h. After addition of NaN<sub>3</sub>/NaF (10 mM of each), proteins were extracted by the rapid protein extraction protocol, separated on an SDS-PAGE gel and detected with anti-Gas1p antibodies and enhanced chemiluminescence. The immature 105 kD and the mature 125 kD form of Gas1p was quantified by densitometry. Relative amounts of the mature form of Gas1p protein are given for every strain. Breakdown products of Gas1p were not taken into account.

### 3.5.3. Discussion

Two screens for the production of temperature-sensitive *gpi8* mutants were performed and identified 10 putative temperature-sensitive *gpi8* mutants. The first screen was based on *in vitro* mutagenesis of *GPI8* with hydroxylamine and plasmid shuffling with transformants of FBY525 followed by growth analysis at 24 and 37 °C. Seven *gpi8* ts mutants, 5, 6, 18, 20, 23, 34, and 41, were identified as strains which grew well at 24 °C, and badly at 37 °C. Mutant 34 which displayed already impaired growth at 24 °C was the only exception. None of these mutants showed strong accumulation of CP2 or retarded maturation of Gas1p as determined by labeling with [2-<sup>3</sup>H]-*myo*-inositol and immunoblotting of cell extracts with anti-Gas1p antibodies, respectively. Furthermore, some of these mutants developed revertants which - together with the incomplete growth defect at 37 °C - led us to the conclusion that none of the mutants was suitable for a synthetic lethality screen. However, recent evidence showed that the growth defect of all these mutants could be complemented either completely in the mutants 18, 20, 23, 34, and 41, or partially in the mutants 5 and 6 by overexpression of wt *GPI8* from the multicopy vector YEp*GPI8*. This indicates that the growth defect in all mutants was due to reduced *GPI8* function.

A second screen was based on the substitution of the endogenous *GPI8* gene by a random PCR mutagenized *gpi8* allele in wt cells. This was realized by transformation of cells with a linearized integrative plasmid containing a non-functional, truncated *GPI8* promoter fused with mutagenized *gpi8* alleles. Growth analysis at 24 and 37 °C led to the identification of three tight temperature-sensitive mutants. The growth defect of mutants 7 and 10 could be completely complemented by overexpression of wt *GPI8* indicating that one or several mutations in the expressed *gpi8* allele were responsible for the complete and rapidly occurring growth defect. This explanation was confirmed by the fact that both mutants strongly accumulated CP2 and immature Gas1p already after 1 h of incubation at 37 °C. The growth defect of mutant 3.1 is more difficult to explain. The screen strategy implies that mutations were only introduced into the *GPI8* gene, however, unprecise integration of the plasmid or the occurrence of an spontaneous mutation could also account for the strong temperature-sensitive growth defect. The growth defect could not be complemented by overexpression of wt *GPI8*, and at the restrictive temperature, mutant 3.1 showed a lipid profile identical to that of wt cells. Nonetheless, this mutant showed a heavily impaired maturation of Gas1p at the non-permissive temperature, a phenotype which nevertheless might be mediated by a strongly dominant-negative *gpi8* allele which does not lead to accumulation of CP2. Sequencing and reintegration of this *gpi8* mutant allele into wt cells might reveal whether the strong growth defect of this clone is due to a mutated *gpi8* allele.

However, mutants 7 and 10 are promising to serve as appropriate tools for a synthetic lethality screen; since their strong growth defect at 37 °C is a direct consequence of a mutated *gpi8* allele, they were renamed as *gpi8*-2 and *gpi8*-3, respectively.

### 3.5.4. Experimental Procedures

General information on strains, plasmids and protocols for the cultivation of yeast cells can be found in chapter 5. Radiolabeling of GPI lipids and immunoblot analysis were performed as described (Meyer *et al.*, 2000; chapter 3.1).

#### *In vitro* mutagenesis with hydroxylamine (Rose and Fink, 1987) and plasmid shuffling

Hydroxylamine mutagenesis was adapted from a protocol described previously (Rose and Fink, 1987). 10 µg of plasmid pBF53 were dissolved in 500 µl of hydroxylamine solution (7 % (w/v), pH 6.5) and incubated at 37 °C for 20 h. The reaction was terminated by the addition of 10 µl of 5 M NaCl, 50 µl of 1 mg/ml BSA, and 1 ml ethanol to precipitate DNA. For complete precipitation, the DNA was incubated at -20 °C for overnight.

The DNA was collected by centrifugation for 15' at 14'000 rpm and 0 °C. The supernatant was removed and the DNA resuspended in 100 µl TE 1×. DNA precipitation was repeated by adding 10 µl of 3 M sodium acetate (pH 7.0) and 250 µl of ethanol (100 %, and incubation for 1 h at -20 °C). After centrifugation, plasmid DNA ready for electroporation was resuspend in 100 µl TE 1×. FBY525 cells were transformed by electroporation as described (Becker and Guarente, 1991). Mutagenesis of plasmid DNA resulted in a two-fold loss in transformation efficiency. Plasmid shuffling was performed as described (Boeke *et al.*, 1987).

Construction of plasmids pBF53 and YE*pGPI8* was described previously (Meyer *et al.*, 2000; Benghezal *et al.*, 1996).

#### *Construction of the gpi8 allele library in plasmid pBF515*

A first version of plasmid pBF515, pBF515h was a ligation product of the 3780 bp *Sma*I/*Sal*II of plasmid pBF511, the 1630 bp *Alw*NI/*Sal*I fragment and a third *Alw*NI/blunt end fragment containing the truncated *GPI8* promoter and the *Eco*NI site. For the *Alw*NI/blunt end fragment, the 4 overlapping oligonucleotides O1 5'-GCTTAGCAAATTGC-AAAAGCCGAACAAAATGCGTATAGCGATG-3', O2 5'-CTTACTACCTCTTT-CAGGTGCAAATAACACAGATGCTGCACACGAAGT-3', O3 5'-CACCTGAAA-GAGGTAGTAAGAATATGTATAAAAGCAACAGCGGC-AGATGCATCGCTATACGCATTTTG-3' and O4 5'-ACTTCGTGTGCAGCATC-3' were annealed and gaps filled up with bacteriophage T4 DNA polymerase resulting in a 120 bp DNA fragment which was cut with *Alw*NI resulting in the *Alw*NI/blunt end fragment. Sequencing of the ligation product revealed that the sequence upstream of the *Eco*NI site was incomplete (plasmid pBF515h). Therefore, the region upstream of the *Eco*NI site was amplified by PCR using primers 1 5'-TCACATGGTACCATAGCAAATTGCAAAA-3' and 2 5'-TTTGCACCTGAAAG-AGGTAGTAA-3' and a 2526 bp *Sma*I/*Sal*II fragment of plasmid pBF53 as template. Primer 1 contained the *Eco*NI site and primer 2 a *Kpn*I site also present in the MCS (multiple cloning site) of pBF515h. The 83 bp *Eco*NI/*Kpn*I fragment of this PCR fragment was then ligated with the 5428 bp *Eco*NI/*Kpn*I fragment of pBF515h which resulted in plasmid pBF515 containing the wt *GPI8* gene under the control of a *GPI8* promoter fragment truncated just after the TATA box.

The pBF515\* library containing a variability for mutant *gpi8* alleles of more than 36'000 was a ligation product of the 4325 bp *Sst*I/*Bst*XI fragment and the 111 bp

SstI/AlwNI of pBF515 and the 1074 bp AlwNI/BstXI fragment of the library YEp*GPI8\**. Control plasmids pBF521 and pBF522 were obtained by ligation of the 1744 bp SstI/SalI fragment of pBF515 with the 4827 bp SstI/SalI fragment of plasmid pBF53 and by ligation of the 819 bp BstXI/SalI fragment of plasmid pBF511 with the 4958 bp BstXI/SalI fragment of plasmid pBF515, respectively.

### 3.5.5. Acknowledgements

We are greatly indebted to the diploma student Serge Summermatter who performed the second screen for temperature-sensitive *gpi8* mutants and Markus Britschgi who produced mutant *gpi8* alleles by random PCR. We are thankful to Dr Markus Aebi for excellent advice for the second screen.

### 3.6. GPI anchoring and the yeast proteome

#### 3.6.1. Computational analysis of the yeast proteome for GPI lipid remodelases

##### 3.6.1.1. Introduction

GPI proteins are covalently attached via their C terminal amino acid to a glycosylphosphatidylinositol moiety. Most of the GPI proteins are located at the cell surface, i.e. anchored to the external leaflet of the plasma membrane or covalently integrated into the cell wall. It can be assumed that probably half of the enzymes involved in elaborating the GPI structure and its attachment to newly synthesized proteins in the ER have been identified (cf. Introduction). One important group of GPI biosynthetic enzymes still unidentified are the GPI lipid remodelases, i.e. proteins catalyzing lipid exchange on the GPI anchor present on a GPI protein. GPI lipid remodeling activity has been identified and localized to the ER and the Golgi apparatus (Sipos *et al.*, 1997). Two very different types of lipid moieties can be found in yeast GPI anchors: ceramide (Cer) and diacylglycerol (DAG). The ceramides are found on the majority of yeast anchors and consist of mainly C18:0 phytosphingosine (PHS) and a C26:0 fatty acid. A smaller part of yeast GPI proteins including Gas1p contain diacylglycerol with a C26:0 fatty acid (Fankhauser *et al.*, 1993). In both types of lipid moieties, diacylglycerol and ceramide, the C26:0 fatty acid may be hydroxylated on C2.

Since complete GPI lipid precursors lack ceramide and C26:0 in their lipid moiety (generally, they contain diacylglycerol with C16 and C18 fatty acids), it is assumed that these lipids have to be introduced at a stage occurring after GPI anchor attachment by lipid remodeling of protein anchors. Thus, at least three different types of GPI remodelling can be proposed: substitution of diacylglycerol by ceramide, acyl group exchange on diacylglycerol and acyl group exchange on Cer. Of course, introduction of very long chain fatty acids (C26) could involve several quite different exchange reactions and acyl chain hydroxylation. In addition, it has been shown that remodeling of GPI anchors and biosynthesis of inositol phosphoceramides are mediated by different enzymes suggesting that GPI-specific remodelases exist (Reggiori and Conzelmann, 1998). Since the phenotype of a GPI lipid remodeling-deficient cell is totally unknown and cannot be predicted easily, it is nearly impossible to think of a simple selection procedure or a simple screen to identify GPI remodeling mutants. Therefore and because the whole genome of *Saccharomyces cerevisiae* had been completely sequenced (Goffeau *et al.*, 1996), we decided to screen the whole yeast genome for GPI lipid remodelases by performing computational homology searches with known proteins described to catalyze biochemical reactions such as acyl transfer or phosphodiesterification which resemble putative remodeling reactions. (Reggiori *et al.*, 1997)

##### 3.6.1.2. Results

In short, the basic concept for this screen designed to identify GPI lipid remodelases can be divided into three steps:

**Step1: Identification of unknown ORFs being possible candidates for a GPI lipid remodelase.** The first step of this remodelase screen consists of the identification of protein sequences of unknown function which show homologies to protein sequences



known to catalyze biochemical reactions similar to that of a remodeling reaction. For example, a protein similar or homologous to the described acyl transferase Faa1p might encode a GPI remodelase involved in C26 fatty acid incorporation; or a protein similar to a known phospholipase C might be a candidate for a DAG:ceramide remodelase. Such proteins are defined as “remodelase-like” in the following. Protein sequences, patterns, motifs and profiles used for homology searches are listed in Table 1 and 2. Table 1 describes complete or fragmentary (consensus sequences/domains) protein sequences which were used to perform BLAST searches in the *Saccharomyces cerevisiae* Genome Database (SGD). Table 2 lists - in addition to other complete and fragmentary protein sequences - improved search tools such as motifs, patterns and profiles which were extracted from the PROSITE database or generated by using www software (cf. Methods; chapter on www sites). Furthermore, certain mutants were also introduced into the collection of interesting ORFs. E.g. it has been shown that GPI anchor attachment mutants showed increased sensitivity towards the dye calcofluor white which is most probably due to decreased incorporation of GPI proteins into the yeast cell wall (Benghezal *et al.*, 1995). Several calcofluor white hypersensitive mutants were isolated and the affected genes have been identified (Lussier *et al.*, 1997; Dr F. Klis, personal communication). The corresponding ORFs were analyzed for homologies to known proteins displaying “remodelase-like” reactions. Furthermore, additional ORFs homologous to inositol-specific phosphatases were included into the selection (Dr Bankaitis, personal communication).

**Step2: Selection of ORFs with a transmembrane domain and an N terminal signal sequence.** Since a putative GPI lipid remodelase is suggested to act within or close to the membrane of the ER or the Golgi apparatus (Reggiori *et al.*, 1997), one can assume that such an enzyme needs either a cleavable or an uncleavable N terminal signal sequence to be directed to the secretory pathway and to contain at least a transmembrane domain which allows stable integration into a membrane. These two additional restrictive criteria were introduced to diminish the number of ORFs selected in step 1. A few clones fulfilling only one of these two criteria were not skipped if they showed high homologies to “remodelase-like” proteins. (Although hardly to envisage, it still might be possible that a GPI lipid remodelase is attached to a membrane by a strong interaction with another integral membrane component or that an N terminal signal sequence is not detected by the software.)

In this second step, additional features such as the presence of motifs, the predicted subcellular localization, the membership in any family or homology domain group, the phenotype of the deletant if described, etc. were also taken into account to finally select for ORFs analyzed in step 3. The 20 “first priority” candidates for a GPI remodelase are shown in Tables 3 and 4, the complete list of all 110 ORFs selected in this step is added to the annex part (cf. Annex 8.2).

**Table 1. Proteins, domains or consensus sequences used for the homology search. Part I** (September 1996)

<b>(phospho)lipase proteins</b>
- human GPI-PLD (2 enzymes)
- GPI-PLC ( <i>T. brucei</i> )
- PI-PLC ( <i>B. thurigiensis</i> )
- PI-PLC (rat mRNA)
- PI-PLC ( <i>S. cerevisiae</i> )
- <i>S. pombe</i> homologue to GPI-PLC PLC ( <i>T. brucei</i> )
- phospholipase D (Spo14p; ; <i>S. cerevisiae</i> )
- triacylglycerol lipase (Tgl1p; ; <i>S. cerevisiae</i> )
- PLA <sub>2</sub> (all described enzymes)
- phospholipase B (lysophospholipase; Plb1p; <i>S. cerevisiae</i> )
- Plb3p (Spo1; YNL012w; <i>S. cerevisiae</i> )
<b>acyltransferase proteins</b>
- 1-acyl-sn-glycerol-3-phosphate acyltransferase ( <i>Cocos nucifera</i> )
- Slc1p ( <i>S. cerevisiae</i> )
- plsCp ( <i>E. coli</i> )
- Faa1p, Faa2p, Faa3p, Faa4p ( <i>S. cerevisiae</i> )
- malonyl-CoA:acyl carrier protein transacylase ( <i>B. subtilis</i> )
- human long chain acyl-CoA synthetase (FACL1; mRNA)
- acyl-CoA synthetase ( <i>S. cerevisiae</i> )
- acyl-CoA:sterol acyltransferase (Sat1p ≡ Are2p; <i>S. cerevisiae</i> )
- acyl-CoA cholesterol acyl transferase (Are1p; <i>S. cerevisiae</i> )
- human PC:sterol O-acyltransferase precursor
- carnitine O-acetyltransferase (Cat2p; <i>S. cerevisiae</i> )
<b>ceramide-binding proteins</b>
- sphingomyelinase ( <i>B. cereus</i> )
- human acid sphingomyelinase
- UDP-galactose:ceramide galactosyl transferase (GCT)
- ceramide glucosyltransferase
- human acid ceramidase
- human cerebroside sulfate activator protein (SAP-MU)
- sphingomyelin phosphodiesterase ( <i>M. musculus</i> )
<b>lipid transport proteins</b>
- Pat1p ( <i>S. cerevisiae</i> )
- Pxa1p ≡ Pat2p ( <i>S. cerevisiae</i> )
- long-chain fatty transporter (fadl; <i>E. coli</i> )
- fatty acid transport protein (FATP; <i>M. musculus</i> )
<b>domains or consensus sequences</b>
- "GPI-PLD consensus sequence" (DVSWHSL)
- Slc1p domain consensus sequence
- β-ketoacyl synthase domain consensus sequence
- domain of ceramide galactosyltransferase
- GPI-binding domain of aerolysine
<b>lipid-modifying enzymes</b>
- lipid elongases
- fatty acid hydroxylases
- acyl-CoA oxidases (Pox1p, Pox2p, Pox4p)

**Table 2. Proteins, patterns, consensus sequences and mutants used for the homology search. PartII** (November 1997)

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**proteins involved in IPC/M(IP)<sub>2</sub>C biosynthesis**

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- Aur1p, Scs7p, Sur1p, Sur2p, YDR072cp

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**known patterns (PROSITE) for lipid-binding proteins**

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- PLA<sub>2</sub> (His and Asp)
- lipolytic enzymes (lipases/esterases)
- acyltransferases
- acyl-CoA binding
- oxidoreductases

**patterns and consensus sequences were produced from ceramide- or GPI-binding proteins (and fragments) in different combinations which were generally common to all and in a few cases common to at least two of the analyzed proteins<sup>¶</sup>**

---

- *ceramide binding motifs*:

- ceramide glucosyltransferase (CEGT), UDP-galactose:ceramide galactosyltransferase (CGT);
- glucosylceramidase (GLCM), cerebroside sulfatase (ARSA), acid sphingomyelinase
- Scs7p, Sur2p, Aur1p

- *GPI binding motifs*:

Gpi3, Gpi10, aerolysin, GPI-PLD, GPI-PLC, Gpi7, Gpi8

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**consensus sequence and pattern for PI-PLC<sup>#</sup>**

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- PI-PLCs from rat, cow, *B. thurigiensis* and *S. cerevisiae*

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**profiles**

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- acyltransferases/YDR018c
- Aur1p/PA phosphatases
- PI-PLC
- carnitine palmitoyl-transferases

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**previously isolated mutants and additional ORFs**

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- ECM mutants being hypersensitive to CFW (41 ECM mutants found in SGD (*Saccharomyces cerevisiae* Genome Database; Lussier *et al.*, 1997).
- CFW hypersensitive mutants obtained from Dr Frans Klis
- ORFs homologous to inositol-specific phosphatases as communicated by Dr Bankaitis

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<sup>¶</sup> It failed to produce high-score patterns since these proteins are apparently not related with each other in terms of aa sequence; their amino acid sequence is most probably rather defined by their function than by their ligand-binding feature; unfortunately, the glycosyltransferase domains of CEGT and CGT are also not related with each other.

<sup>#</sup> PI-PLCs are a very specific family of enzymes, they appear not to be related to other enzymes catalyzing comparable chemical reactions (phosphatases, PLDs, etc.). With a PI-PLC pattern, only PI-PLCs are found.

**Step3: Biochemical analysis of GPI remodelase activity in deletants.**

Deletant strains or mutants of the ORFs selected in step 2 were obtained from a lot different sources including EUROFAN 1 members, the EUROSCARF deletant strain collection, Research Genetics and independent research groups. These strains were then analyzed for loss of GPI remodelase activity by several people of our lab as basically described (Sipos *et al.*, 1997; Reggiori and Conzelmann, 1998; Reggiori *et al.*, 1997). ORFs in the first part of Table 3 were analyzed by Dr Fulvio Reggiori (now at Cambrigde, UK) and analysis of both acyl chain and diacylglycerol→ceramide remodeling in the corresponding deletants gave no differences compared to wt strains (Fulvio Reggiori, personal communication). Biochemical analysis of 50 additional deletants was continued in the context of the EUROFAN 2 project by Isabelle Guillas, Martin Pfefferli, and Carole Roubaty. Certain mutants showed altered levels of IPC/C, IPC/B or PI (EUROFAN 2: Final report of activities of group of Andreas Conzelmann, Fribourg, Switzerland; [http://www.mips.biochem.mpg.de/proj/eurofan/eurofan\\_2/n7/index.html](http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_2/n7/index.html)). However, the observed variations do not prove the presence of elevated or reduced amounts of a given remodelase.

**Table 3. First priority search results obtained by BLAST searches as listed in Table 1.** Proteins denoted unknown in the databases MIPS and YPD (January 1998).

<b>YJL132W: Chrs. X; protein: S55180 (750 aas)</b>	
•	weak similarity to human PLD
•	homologous to human GPI-PLDs
•	homologous to bovine GPI-PLD
•	shares the putative motif DVSWHSL with the two human GPI-PLDs (this motif has been found in two other possible <i>S. cerevisiae</i> proteins which are both unknown: S53585 (YAL06 (PIR), 126 aas); P32613 (YEF8_YEAST (SwissProt), 152 aas));
	this motif is also present in human PLD; in bovine GPI-PLD you find DVNWHSL
•	it is not a member of any protein family or homology domain group
•	putative transmembrane protein (1 TM domain predicted)
•	seems to have a cleavable N-terminal signal sequence
•	predicted localization (PSORT): outside>vacuole>peroxisome>ER
<b>YDR018C: Chrs. IV; protein: S54641 (396 aas)</b>	
•	homologous to 1-acyl-sn-glycerol-3-phosphate acyltransferase ( <i>Cocos nucifera</i> )
•	weakly homologous to Slc1p
•	strong similarity to hypothetical protein YBR042C (cf. second priority proteins)
•	it is not a member of any protein family or homology domain group
•	putative transmembrane protein (3 TM domains predicted); most likely a Type IIIb membrane protein (Nexo C <sub>Cyt</sub> ), favored for ER or Golgi localization
•	seems to have no N-terminal signal sequence, but this protein has a KKXX motif
•	predicted localization (PSORT): plasma membrane>Golgi>ER or peroxisome
<b>YNL101W: Chrs. XIV; protein: Z71377 (713 aas)</b>	
•	weak homology to Faa4p and to Are1p
•	similar to members of a putative transporter family (YBR089*, YER064*, YER119*, YIL088*, YKL146*, YNL101*)
	no further information has been found about the possible function of these gene

- products (not found as homologs of lipid-binding enzymes)
- it is not a member of any protein family or homology domain group
- putative transmembrane protein (9 TM domains predicted); most likely a Type IIIa membrane protein (N<sub>cyt</sub> C<sub>exo</sub>),
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): plasma membrane>Golgi>ER or peroxisome

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**YLR020C Chrs. XII; protein: Z73192x1 (538 aas)**


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- similar to triacylglycerol lipase (Tg11p)
- putative transmembrane protein (1 TM domain predicted); seems to be a Type Ib membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have an uncleavable N-terminal signal sequence; eventually intramitochondrial signal sequence
- predicted localization (PSORT): ER (mb) >> perox. > PM > ER (lumenal)

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**YLL048C Chrs. XII; protein: Z73153x1 (1661 aas), identified: vacuolar protease**


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- similar to Pat1p and Pat2p
- homologous to putative transport proteins YHL035C and YKR103W
- putative ABC transporter, has an ATP/GTP-binding site motif A (P-loop)
- putative transmembrane protein (12 TM domains predicted); seems to be a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have no N-terminal signal sequence; C terminus is KKD (?)
- predicted localization (PSORT): PM > Golgi > ER or perox.

---

**YDR452W: Chrs. IV; protein: U33007\*37 (674 aas)**


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- similar to human acid sphingomyelinase (produces ceramide and phosphocholine, PLC-like) and sphingomyelin phosphodiesterase (human and M. musculus, PLD-like?)
- it is not a member of any protein family or homology domain group
- putative transmembrane protein (1 TM domain predicted); seems to be a Type II membrane protein (N<sub>cyt</sub> C<sub>exo</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): mitochondrial inner membrane>plasma membrane>nucleus>Golgi

---

**YHR080C: Chrs. VIII; protein: P3880 (1345 aas),**


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- homologous to Slc1p-derived domain consensus sequence
  - similar to hypothetical proteins YDR326C (transmembrane), YFL042C (no TM) and YLR072W (transmembrane)
  - ATP/GTP binding site
  - putative transmembrane protein (1 TM domain predicted);
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus>peroxisome>mitochondrial matrix>ER
-

**Table 4. First priority search results obtained by homology searches as described in Table 2.**

Proteins denoted unknown in the databases MIPS and YPD (January 1998).

<b>1. homologous to acyltransferase/YDR018c profile</b>	
<b>YPR140w: Chrs. XVI; (381 aas), 814386-815528</b>	
•	similar to human Barth syndrome gene taffazin
•	no TM domain predicted, but has a KKKXX motif
•	seems to have no N-terminal signal sequence
•	predicted localization (PSORT): peroxisome>mitoch. matrix
<b>YBL011w: Chrs. XIII; (759 aas); SCT1, 203495-105771</b>	
•	suppressor of the choline transport mutant ctr1
•	3 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
•	seems to have no N-terminal signal sequence
•	predicted localization (PSORT): PM>Golgi, ER
<b>YKR067w: Chrs. XI; (743 aas), 567557-569785</b>	
•	similar to Sct1p
•	5 TM domains predicted; Type IIIb (Nexo Ccyt) which is favored for ER mb; has KKX at the very C terminus (?)
•	seems to have no N-terminal signal sequence
•	predicted localization (PSORT): PM>Golgi, ER
<b>2. Homologous to Aur1p/PA phosphatase profile</b>	
<b>YJL134W: Chrs. X; (409 aas); LCB3</b>	
•	involved in incorporation of exogenous long chain bases in sphingolipids; KO is viable and has a reduced rate of incorporation of long chain base into sphingolipids, but YKR053 (to which LCB3 is similar) null mutant does not; KO is more resistant to growth inhibition by long chain bases; KO is more resistant to canavanine and hygromycin B; lcb3/ybr053c double null mutations have no synergistic effect; putative GPI anchor attachment site; putative transporter of sphingoid long chain bases
•	4(-8) TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
•	seems to have no N-terminal signal sequence
•	predicted localization (PSORT): PM>Golgi, mitoch. IM, ER
<b>YKR053c: Chrs. XI; (404 aas), 534921-533710</b>	
•	similar to Lcb3p; KO is viable; not redundant with Lcb3p (cf. YJL134w); KO has the same sensitivity to growth inhibition by long chain bases, canavanine and hygromycin B as wild-type cells; putative GPI anchor attachment site;
•	5 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
•	seems to have no N-terminal signal sequence
•	predicted localization (PSORT): PM>Golgi, ER
<b>YGR036c: Chrs. VII; (239 aas), CWH8, 558862-558146</b>	
•	identical to CWH8, Calcofluor White hypersensitive
•	contains 3 stretches of amino acids that are characteristic for a wide variety of phosphatases, including lipid phosphatases and a protein phosphatase
•	ko viable, severely affected growth rate, hypo-N-glycosylation of secretory

- proteins, severely reduced levels of dolichol-linked oligosaccharides
- similar to *Treponema denticola* phosphatase
- 4 TM domains predicted; Type IIIb (Nexo Ccyt) which is favored for ER mb; has a KXKXX motif
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM, Golgi, ER

---

**YDR503c: Chrs. IV; (274 aas), 1455869-1455048**


---

- related to YDR284c (DAG pyrophosphate phosphatase)
  - similarity to unknown *C. elegans* protein 8U28738\*5
  - 5 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): ER, PM>Golgi
- 

**3. homologous to neutral SMase**


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**YER019W: Chrs. V; (477 aas); 192796-194226**


---

- found as homolog of mammalian nSMase which cuts sphingomyelin and also PC (remaining activity: 30 %)
  - has typical ATP/GTP-binding sites
  - KO is viable
  - 2 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>Golgi, ER
- 

**4. lipase/esterase/thioesterase**


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**YFL025c: Chrs. VI; (1029 aas), BST1 87232-84146**


---

- has the PROSITE motif PDOC00110 (PS50187) at position 136-251 [LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-G-[GSTAC] with the serine as active site
- 10 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM>mitoch., Golgi
- BLAST (NCBI): has unknown homologs

**YOR022c: Chrs. XV; (715 aas); 375856-373712**


---

- has the PROSITE motif PDOC00110 (PS50187) at position 494-516
- has no TM domain, but a KXKXX motif
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): mitoch.
- BLAST (NCBI): mouse membrane-bound associated PI transfer protein, probable calcium transporter

**YOR059c: Chrs. XV; (450 aas); 440259-438910**


---

- has the PROSITE motif PDOC00110 (PS50187) at position 6-105
- P loop (ATP/GTP)
- 1 TM domain predicted; Type IIa (Ncyt Cexo) which is favored for ER mb when the relative position of the cytoplasmic tail is bigger than 30 % (here 59 %); has HKXX at the very C terminus (?)
- seems to have an uncleavable N-terminal signal sequence

- predicted localization (PSORT): ER>PM, Golgi
- BLAST (NCBI): has unknown homologs (YGL144c, YDL109c, etc.)

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**YBR204c: Chrs. II; (375 aas); 633335-632211**


---

- has the PROSITE motif PDOC001 10 (PS50187) at position 87-192
  - similar to peroxisomal serine-active site lipase
  - 1 TM domain predicted; Type Ib (Nexo Ccyt)
  - has a C-terminal SLK peroxisomal targeting signal
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): Perox. > ER
  - BLAST (NCBI): putative triacylglycerol lipase 2 in *Mycoplasma pneumoniae*
- 

**5. ORFs of calcofluor white hypersensitive mutants**


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**YML048w: Chrs. XIII; (403 aas); ECM6, EFF2 178426-179634**


---

- plays a role in glucose repression, synergistic effect with Gsf1p on SUC2 suppression
- 1 TM domain predicted; Type II (Ncyt Cexo); has a KKXX motif
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): ER
- BLAST (NCBI): homologous to unknown proteins

**YJR106w: Chrs. X (725 aas); ECM27 624525-626699**


---

- 12 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
- seems to have no N-terminal signal sequence
- weak similarity to acylglycerol lipase YJR107w
- predicted localization (PSORT): PM, Golgi, ER
- BLAST (NCBI): homologous to unknown protein in *C. elegans*, similar to sodium/calcium potassium exchanging protein (antiporter)

**YOL025w: Chrs. XV (680 aas); ECM36/LAG2**


---

- protein involved in longevity
- 1 TM domain predicted
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): mitoch.
- BLAST (NCBI): no homologs

**YBR078w: Chrs. II, (468 aas); ECM33**


---

- hypersensitive to CFW, zymolase, hygromycin, killer toxin, decreased level of mannose to glucose in the cell wall
- 2 TM domains predicted; likely type IIIa
- seems to have a cleavable N-terminal signal sequence
- predicted localization (PSORT): PM, Golgi, ER

**YAL059w: Chrs. I, (212 aas); ECM1**


---

- probably a TMD protein
- seems to have an uncleavable N-terminal signal sequence
- predicted localization (PSORT): ER

**YBR162c: Chrs. II; (455 aas)**


---

- similar to Aga1p, Yil171p, Tir2p, about 10 mRNA copies per cell



- ko viable
- 1 TM domains predicted
- seems to have a cleavable N-terminal signal sequence
- predicted localization (PSORT): outside, ER, Golgi

---

**YNL058c: Chrs. XIV; (316 aas)**


---

- similarity to Yil117p, has a putative PEST motif
  - 1 or 2 TM domains predicted; Type Ia
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM, (ER, outside)
- 

### 3.6.1.3. Discussion

Several possible candidates for a GPI lipid remodelase were identified by computational analysis of all non-redundant translated ORFs of the yeast *S. cerevisiae* genome. The selection procedure described in the Results section bases on several assumptions which might be more or less suitable for such a screen. It has to be questioned whether a GPI lipid remodelase really shows homologies or similarities to the proteins we have chosen as search tools. Eventually GPI lipid remodelases may define a very specialized subgroup of lipid modifying enzymes as distinct from all the other as it has been found for example for the PI-PLC family (Table 2).

In addition, because no GPI remodelase had been identified so far, the homology search was designed to search rather for similarities (or low homologies) than for the identification of yeast homologs of certain lipid modifying enzymes described in other species; a circumstance which makes such a selection quite empirical. Very useful tools for such an empirical search are profiles and - to a smaller extent - patterns and motifs as used in the second part of the search (Table 2). The structure of proteins is strongly influenced by their enzymatic activity and their ligand binding capacities. We could generate quite promising profiles and motifs from proteins, the structure of which was seemingly highly specific for their enzymatic activity, but we failed or obtained low score results when we tried to generate ligand binding profiles such as for example a profile for GPI lipid binding or ceramide/sphingolipid binding. Of course, this might be also be due to the fact that we had only little structural and biochemical information on the proteins used to search the databases. However, increasing knowledge on proteins involved in GPI anchor biosynthesis and the use of newly identified lipid modifying enzymes might help to design better profiles for our remodelase screen. For example, analysis and aligning of 19 PLA<sub>2</sub> proteins of different organisms which gave no well conserved pattern, but formed several subgroups which let us identify several new potential yeast GPI lipid remodelase ORFs. Moreover, the ORFs found in this screen may help to identify other proteins required for lipid metabolism.

Although no ORF could be identified biochemically as being involved in GPI lipid remodeling, work on several of the anomalous mutants is continued in the lab in an attempt to explain their abnormal lipid profiles.

### 3.6.1.4. Methods

URLs of all databases, molecular biology servers and sequence analysis tools are listed in the www sites chapter. References are cited on the web pages. Sequences were retrieved from SGD (*Saccharomyces cerevisiae* Genome Database), YPD (Yeast Protein Database), MIPS (Munich Information center on Protein Sequences), SwissProt (Swiss Protein Database) and TrEMBL (Translated EMBL). Sequence alignments of the first part of the remodelase screen (Table 1) were performed with the BLASTp algorithm (Altschul *et al.*, 1996) on the molecular biology servers at SGD, NCBI and SIB. ClustalW multiple sequence alignments were done at EBI (European Bioinformatics Institute server) or the Multiple Sequence Alignment processor of the Bork lab (EMBL, Heidelberg). Motifs were generated based on multiple sequence alignments by using the Blocks Multiple Alignment Processor and used for database searching with the Software therein (MAST (multiple alignment sequence tool)). Other motifs were made, searched and scanned with the emotif software (Stanford). Patterns were retrieved from the PROSITE database or generated by using the PRATT program at EBI and employed as search sequence ScanProsite2 software. Profiles were retrieved from the profile database at ISREC (Swiss Institute of Experimental Cancer Research) or generated on the local ISREC server with the kind support of Dr Kay Hoffmann and used for database analysis by using the ProfileScan software. Profile-like searches were executed by using the  $\psi$ -BLAST at SIB and NCBI. Protein families and domains were analyzed with the Pfam database and the ProDom software, respectively.

Basic information on the selected ORFs with unknown function was retrieved mainly from the databases YPD, SGD and MIPS. The presence of transmembrane domains and of N terminal signal sequences as well as the subcellular localization were predicted with the PSORT software. Further analysis was carried out by using protein sequence analysis software from ExPASy.

Deletant or other mutant strains were obtained from members of the EUROFAN 1 (European Functional Analysis Network 1) project, EUROSCARF (*European Saccharomyces Cerevisiae* Archive for Functional analysis), and independent research groups as will be cited.

### 3.6.2. A relational database for yeast GPI proteins

#### 3.6.2.1. Introduction

Since the GPI signal sequences (GPI-SSs) generally lack a consensus sequence and their similarity among each other is defined rather by overall physico-chemical properties, it remains difficult to predict precisely all yeast GPI proteins although the whole genome of *Saccharomyces cerevisiae* has been completely sequenced already more than 4 years ago (Goffeau *et al.*, 1996). Indeed, two different approaches to predict GPI proteins by analyzing all non-redundant open reading frames (ORFs) of *Saccharomyces cerevisiae* gave different results, precisely because different criteria were chosen. On the one hand, Caro *et al.* (1997) predicted GPI proteins by screening all yeast non-redundant ORFs for the presence of an N terminal signal sequence using the Von Heijne algorithm (1983) which gave 686 proteins and among them, selected for the presence of a GPI-SS as defined by Udenfriend and Kodukula (1995); determination of the  $\omega$  site was performed using the algorithms of Udenfriend and Kodukula (1995) and Nuoffer *et al.* (1993). On the other hand, Hamada *et al.* (1998) identified putative GPI proteins based on three criteria: the presence of a GPI-SS, the presence of signal sequence for secretion and a serine- or threonine-rich sequence, the last criterion considered as being specific for GPI-dependent cell wall proteins. The hydrophobicity of the last 20 C terminal amino acids was calculated by using the method of Kyte and Doolittle (1992) and yielded 454 ORF. These ORFs were screened for signal sequences using the Von Heijne algorithm (1983), and subsequently for the presence of serine/threonine-rich sequences. These calculations identified 70 ORFs; the hydrophobicity of their C terminal sequences were compared with those of the known GPI proteins Ag $\alpha$ 1p, Sed1p, Gas1p, and Yap3p which resulted in the disqualification of 17 ORFs. Since the criterion for the presence of a GPI-SS was different from that chosen by Caro *et al.* (1997), additional ORFs were identified by this approach. Furthermore, the yeast protein database (YPD) provides a list of 33 GPI proteins. Thus, information on yeast GPI proteins is to some extent dispersed which partially complicates their analysis. In order to generate an overview on all yeast GPI proteins and in order to ease simple and more exacting exploration of them, a relational database was designed.

#### 3.6.2.2. Results

##### *Building the yeast GPI protein database*

A relational database containing both biochemically verified and predicted GPI proteins was created by using the Microsoft Access 97™ software (Meier, 1998a). The database was designed for two major purposes: first, to collect general but rather GPI-specific information on yeast GPI proteins including the complete amino acid sequence and the annotation of the  $\omega$  site, and the subcellular localization; second, to answer precise questions with the main focus on the analysis of the GPI signal sequence (GPI-SS) of all yeast GPI proteins or subgroups of them.

The database was built by following the rules for relational database design (Meier, 1998b). Yeast GPI proteins were extracted from three sources: Caro *et al.* (1997), Hamada *et al.* (1998) and the Yeast Protein Database (YPD, 1.09.2000), which described 58, 53 and 33 GPI proteins. Combining of all these data gave a putative set

of 70 ORFs encoding biochemically verified or predicted yeast GPI proteins. GPI attachment sites ( $\omega$  sites) were extracted from the above mentioned sources or determined by the big-PI predictor (Eisenhaber *et al.*, 1998; for www sites cf. chapter 7). A list of all 70 putative yeast GPI proteins with the corresponding ORF, name and the  $\omega$ -site region can be found in the annex part (chapter 8.3).

The following data sets were introduced for all 70 predicted yeast GPI proteins: the yeast systematic ORF, the SwissProt accession number, the mostly used name, the alternative names, information about the modification by a GPI anchor, the subcellular localization, the complete amino acid sequence, information on the GPI signal sequence such as the position of the known or predicted  $\omega$  site and its length, the quality of the  $\omega$  site determination. In addition the database contains a set of amino acid features such as the written name, the abbreviations in one- and three-letter code, the volume, the occurrence, and several properties of their side chains which can be used as parameters to analyze the GPI signal sequence. The database was designed in a way that sequence queries for the GPI signal sequence can be performed more efficiently than queries for the N terminal rest of the filed GPI protein sequence. The database allows simple and combined queries by applying the widely-used query language SQL (Structured Query Language) as well as by Access97-specific queries. Standard queries are implemented in SQL as for example for the properties of the  $\omega$  site position. A more precise description of the database is in preparation (cf. my diploma thesis in information systems entitled "A relational database for GPI proteins", Institute of Informatics, University of Fribourg).

### *Preliminary queries*

Although the database was still under construction, several preliminary queries were performed to test its functionality. First of all, it is of interest to know how many of the 70 predicted GPI proteins were experimentally shown to be modified by a GPI anchor. For that, GPI proteins previously described to be experimentally verified (Caro *et al.*, 1997; Hamada *et al.*, 1998; YPD, 2000; and references therein) were combined with the GPI modification data published by Hamada *et al.* (1998); in this study,  $\alpha$ -galactosidase constructs were fused to the 40 C-terminal amino acids of predicted GPI proteins and treated by PI-PLC in order to assess modification by a GPI anchor. As can be seen in Table 1, 50 out of these 70 proteins were experimentally shown to be GPI-anchored. For all selected proteins, the experimentally shown or predicted  $\omega$  site position described in the cited articles is listed together with the positions  $\omega+1$  and  $\omega+2$ . For the ORFs YCR098c/GIT1, YDR134c, YGL259w/YPS5, YLR046c, YGR014w, YIR039c/YPS6 and Sta1, the  $\omega$  site was predicted by using the big-PI Predictor ([http://mendel.imp.univie.ac.at/gpi/gpi\\_server.html](http://mendel.imp.univie.ac.at/gpi/gpi_server.html)). For Sta1, no systematic gene name was indicated, its gene was only found in the variant *S. cerevisiae* var. *diastaticus* and is thought to result from a recombination between SGA1 and FLO11 (Lo and Dranginis, 1996).

**Table 1. Experimentally verified GPI proteins and their omega site region**

ORF	Name	$\omega$	$\omega+1$	$\omega+2$
YAL063c	Flo9	G	S	A
YAR050w	Flo1	G	S	A
YBR067c	Tip1	N	A	G
YCR089w	Fig2	G	S	A
YCR098c	Git1	N	G	T
YDR055w		N	A	A
YDR077w	Sed1	N	G	A
YDR134c		S	P	A
YDR144c	Mkc7	N	G	G
YDR261c	Exg2	N	A	A
YDR349c	Yps7	G	A	G
YDR534c		G	A	A
YEL040w	Utr2	N	A	G
YER011w	Tir1	G	A	A
YER150w	Spi1	N	A	A
YGL259w	Yps5	S	A	L
YGR014w	Msb2	S	S	S
YGR189c		N	G	A
YHR211w	Flo5	G	S	A
YIR019c	Muc1	G	A	A
YIR039c	Yps6	N	A	G
YJL078c	Pry3	G	A	A
YJL171c		N	G	V
YJR004c	Sag1	G	K	A
YJR150c	Dan1	N	G	A
YJR151C		N	G	A
YKL096w	Cwp1	N	A	G
YKL097w	Cwp2	N	G	A
YKR102w	Flo10	G	I	A
YLR042c		G	V	G
YLR046c		D	K	S
YLR110c		G	A	A
YLR120c	Yap3	N	V	G
YLR121c	Yps3	N	S	A
YLR391w-a	Ssr1	N	V	L
YMR008c	Plb1	N	A	G
YMR215w	Gas3	N	V	S
YMR307w	Gas1	N	A	A
YNL190w		N	A	A
YNL300w		G	A	A
YNL322c	Kre1	N	E	A
YNL327w	Egt2	G	A	A
YNR044w	Aga1	G	S	G
YOL011w	Plb3	S	H	L
YOR009w		N	G	A
YOR010c	Tir2	N	G	A
YOR214c		N	S	S
YOR382w		G	A	A
YPL130w	Spo19	N	A	S
	Stal	A	F	N

It is thought that yeast contains two groups of GPI proteins: one group consists of plasma membrane-resident proteins such as Gas1p (Conzelmann *et al.*, 1988b) or Yap3p (Ash *et al.*, 1995), and the other group consists of proteins such as  $\alpha$ -agglutinin (Chen *et al.*, 1995) or Cwp2p (van der Vaart *et al.*, 1995) which have a part of their anchor cleaved and then get covalently linked to the cell wall. A query for subcellular localization among the combined data found in the 3 basic sources (Caro *et al.*, 1997; Hamada *et al.*, 1998; YPD, 2000; and references therein) resulted in the listing of 27 cell wall and 5 plasma membrane yeast GPI proteins (Table 2). All these proteins have of course also been shown experimentally to be modified by a GPI anchor and are listed in Table 1.

**Table 2. Yeast GPI proteins with experimentally verified subcellular localization**

Cell wall		plasma membrane	
ORF	Name	ORF	Name
YAL063c	Flo9	YDR144c	Mkc7
YAR050w	Flo1	YDR261c	Exg2
YBR067c	Tip1	YLR120c	Yap3
YCR089w	Fig2	YMR307w	Gas1
YDR077w	Sed1	YNL322c	Kre1
YDR134c			
YDR534c			
YEL040w	Utr2		
YER011w	Tir1		
YER150w	Spi1		
YGR189c			
YHR211w	Flo5		
YIR019c	Muc1		
YJL078c	Pry3		
YJR004c	Sag1		
YJR151C			
YKL096w	Cwp1		
YKL097w	Cwp2		
YKR102w	Flo10		
YLR110c			
YLR391w-a	Ssr1		
YNL300w			
YNL327w	Egt2		
YNR044w	Aga1		
YOR009w			
YOR010c	Tir2		
YOR214c			

A final query shows the combination of basic data on yeast GPI proteins with an amino acid property. The query asks for experimentally verified GPI proteins which have at the  $\omega$  site an amino acid containing an amide group in the side chain and which is localized to the cell wall (Table 3). The results are listed with the SwissProt accession number together with the  $\omega$  site in one-letter code and can be verified by combining the results from Table 1 and 2.

**Table 3. Experimentally verified yeast GPI proteins having an amide group-containing  $\omega$  site and are localized to the cell wall.**

ORF	Name	SwissProt	$\omega$	Localization
YBR067c	Tip1	P27654	N	cw
YDR077w	Sed1	Q01589	N	cw
YEL040w	Utr2	P32623	N	cw
YER150w	Spi1	P40092	N	cw
YGR189c		P53301	N	cw
YJR151C		P47179	N	cw
YKL096w	Cwp1	P28319	N	cw
YKL097w	Cwp2	P43497	N	cw
YLR391w-a	Ssr1	O13547	N	cw
YOR009w		Q12218	N	cw
YOR010c	Tir2	P33890	N	cw
YOR214c		Q12282	N	cw

### 3.6.2.3. Discussion

A few queries showed that the database stores reliably information and treats otherwise time-consuming queries in an efficient manner. However, the database is still under construction and the information entered into the database is still far from being satisfactory, which means that answers on database queries should still be considered as tentative. Moreover, the database does not yet contain a complete annotation of references. The  $\omega$  position not specified for a subset of predicted or known GPI proteins was determined by using the big-PI Predictor software. Of course, it has to be considered that the listed  $\omega$  sites as predicted by the big-PI Predictor software as well as by comparison with biochemically determined sequences are not faultless and should be used cautiously. As for example, the  $\omega$  site region of YDR134c predicted by the big-PI Predictor is most probably wrong since it contains a proline at the  $\omega+1$  position which has been shown - at least for Gas1p - to not be allowed (Nuoffer *et al.*, 1993). Generally speaking, the criteria which define the  $\omega$  site in yeast as well as in other organisms are not so restrictive as that they would allow very high-probability predictions. In addition, it still might be possible - even if it is considered that in general biochemical reactions in living systems are very specific - that the GPI:protein transamidase is able to add a GPI anchor to more than one amino acid position of the same protein (Aceto *et al.*, 1999).

Subcellular localization has been published for several yeast GPI proteins and was introduced into the database. These descriptions have to be considered cautiously since for certain GPI proteins subcellular localization data are conflicting. As for example, the well-known GPI-anchored model protein Gas1 published some time ago to be present the plasma membrane (Nuoffer *et al.*, 1993) was recently extracted by glucanase treatment of yeast cell walls which had been extracted twice with SDS before (De Sampaio *et al.*, 1999). Furthermore, antibodies might show different affinities for plasma membrane and cell wall forms of a GPI protein.

A simple combined query (Table 3) has shown that the GPI protein database might help to answer certain basic questions such as for example, whether certain GPI-SS features define the subcellular localization of a GPI-anchored protein.

#### **3.6.2.4. Methods**

For database design, data types were organized by establishing an entity-relationship model (ERM) which was then translated into a relational database as described (Meier, 1998a). The database was implemented by using the software of Microsoft® Access 97 (for details: cf. diploma thesis in information systems, A relational database for GPI proteins, Institute of Informatics, University of Fribourg). For this first version of the yeast protein database, data were basically extracted from three sources (Caro *et al.*, 1997; Hamada *et al.*, 1998; YPD, 2000). All queries were programmed by using the query language SQL (Date and Darwen, 1993). A web-based version of the database is under construction.



## 4. FINAL DISCUSSION

### 4.1. Conclusions

As described in chapter 2, the major goal of this thesis was the further characterization of the yeast *Saccharomyces cerevisiae* Gpi8p protein and of its role in the process of GPI anchor attachment. Analyses done during the thesis provide evidence for the presence of an active site as being a diad of amino acid residues C199 and H157. Homologies in terms of similar amino acid sequences around these active site residues with members of two cysteine protease families, the C13 family of plant cysteine proteases, and the C14 family of caspases confirm the importance of these residues. Gpi8p thus uses a well conserved catalytic mechanism to cleave a peptide bond by forming a intermediate thioester linkage with the carbonyl group of the corresponding peptide bond. However, a direct role of Gpi8p in the cleavage of the GPI signal sequence is still not proven.

Up to now, no proteolytic activity could be found with purified Gpi8p in an *in vitro* system (Benghezal *et al.*, 1996; Fraering *et al.*, submitted). We tried to find *gpi8* mutants which show proteolytic activity *in vivo* by using a GPI reporter protein which is generally GPI anchored and becomes covalently integrated into the cell wall. Although more than 27'000 different clones were tested and several mutants were identified which show a dominant-negative effect, no proteolytic Gpi8p mutant could be discovered. This failure might be due to technical flaws, but it also is possible that members of the *GPI8* subfamily became so highly specialized during evolution that neither wt nor mutant Gpi8p can display proteolytic activity any more.

Further experiments addressed the role of Gpi8p in the recognition of the GPI-SS. De Sampaio *et al.* (1999) published that a fusion protein consisting of  $\alpha$ -galactosidase and the GPI-SS of human placental alkaline phosphatase (hPLAP) is only processed by the yeast GPI anchoring machinery when the human Gpi8p is co-expressed. This indicated that Gpi8p plays a decisive role in the recognition of the GPI-SS sequence. By using appropriate controls, we show in contrast to the results published by De Sampaio *et al.* (1999) that the GPI-SS sequence of hPLAP is not recognized by the yeast GPI anchoring machinery even when human Gpi8p is co-expressed suggesting that other factors than Gpi8p are involved in the specific recognition of a GPI signal sequence. Our results are confirmed by the analysis of the maturation of Gas1p mutants which have an impaired GPI signal sequence. Overexpression of yeast Gpi8p does not show a significantly improved maturation of these mutants. Improvement of maturation for these mutants was observed when Gaa1p was overexpressed (Hamburger *et al.*, 1995); co-expression of Gaa1p and of Gpi8p did not increase the positive effect of Gaa1p on maturation of GPI-SS mutants of Gas1p. This analysis speaks in favor of Gaa1p rather than Gpi8p to be responsible for specific recognition of the GPI-SS.

Overexpression studies with dominant-negative *gpi8* active site mutants suggested that Gpi8p might be a subunit of a putative GPI:protein transamidase complex. Further indications for the presence of such a complex come from the co-immunoprecipitation analysis published by Ohishi *et al.* (2000). Human Gaa1p and Gpi8p could be co-immunoprecipitated when both proteins were overexpressed. Furthermore, Fraering *et al.* (submitted) isolated a high molecular complex by blue native gel electrophoresis which contains Gpi8p. Up to now, it is not known whether

this complex contains also Gaa1p and other proteins or whether it is a homo-oligomeric complex of Gpi8p. The identification of several *gpi8* mutants with a temperature-sensitive phenotype for growth could support the ongoing work in our laboratory for the isolation of possible additional components of the putative transamidase complex. Two mutants, *gpi8-2* and *gpi8-3*, show an immediate and strong growth defect at the restrictive temperature concomitant with a strong accumulation of the CP2 lipid and the immature ER form of Gas1p. These mutants could serve as a promising tool in a screen for synthetic lethals, thus offering a tool to isolate new genes which may be involved in the process of GPI anchor attachment. In a first screen which was conceptually not perfect, but easy to perform, genes were identified which are multicopy suppressors of the growth arrest induced by overexpression of the active site mutant C199A of Gpi8p. At the moment, it cannot be definitely excluded that one of the isolated ORFs encodes for a protein involved in GPI anchoring.

Moreover, a small reliable and easy-to-handle relational database was designed for collecting information on yeast GPI proteins. Most of the data on these proteins were dispersed over several publications so far and the exploration of precise questions as for example in terms of the nature of the yeast GPI-SS were time-consuming. Most of the questions can be efficiently extracted by short SQL statements. However, information present in the database is still far from being complete.

Finally, a time-consuming screen for possible candidates of putative remodeling enzymes was performed by homology searches with sequence analysis software accessed by the world wide web. Biochemical study of the corresponding knock out strains or mutants will reveal whether our concepts and features predicted for a putative remodelase were correct or not.

## 4.2. Outlook

The two most important questions which should be addressed to better understand the process of GPI anchor attachment are :

- 1) Are other proteins than Gaa1p and Gpi8p directly involved in GPI anchor attachment? And if this would be the case, which ones?
- 2) What is the exact role of every subunit of the putative GPI:transamidase complex in terms of GPI anchor addition and in terms of interaction with other components of the secretory pathway?

For the first question, several screens could be used to identify additional components of the yeast GPI:protein transamidase complex including co-immunoprecipitation studies with Gpi8p and Gaa1p, isolation of the physiological complex and identification of other subunits by microsequencing, a Two-Hybrid screen or classical genetic screen such as a screen for synthetic lethals. The *gpi8* ts alleles isolated in this study might serve as tools in a synthetic lethality screen to discover other subunits of the putative GPI:protein transamidase.

For the second question, several mutants were identified in this study which might help to understand the function of Gpi8p. Overexpression of dominant-negative alleles of *GPI8* in wt cells and *gpi8* ts mutants at the restrictive temperature show a strong and immediate GPI anchoring defect, whereas other mutants such as S60A when overexpressed in wt cells, or expressed in  $\Delta gpi8$  cells show only a partial but

clearly detectable defect of GPI anchor attachment. Microsomal *in vitro* studies rather than reconstitution systems will help to analyze GPI anchor addition in more detail since the exact requirements for this process are not yet known. Crystallization of Gpi8p alone or together with substrates or other transamidase components such as Gaa1p might increase knowledge on substrate recognition and catalysis of the transamidation reaction. The identification of the active site and the similarity to the active site of caspases, a class of proteases of which at least two members were crystallized together with an inhibitor might help to define working hypothesis. Furthermore, co-immunoprecipitation studies could show a direct interaction of immature GPI proteins with subunits of the transamidase and eventually also help to define the sequence of steps that an immature protein has to go through to become modified by a GPI anchor. And finally, such studies also might reveal interactions of the transamidase and of its substrates with other components of the secretory pathway including the translocation pore complex, chaperones, proteins involved in ER degradation and so on.

Concerning the analyses made in this study, additional experiments should be performed to analyze whether the ER degradation pathway is involved in scavenging GPI proteins which were not modified by a GPI anchor since the fate of unprocessed GPI proteins is not yet completely elucidated although proteasomes were described to be involved in the degradation of a hGF/DAF mutant fusion protein (Wilbourn *et al.*, 1998). Furthermore, it would be interesting to find more evidence relating to the question whether Gaa1p and not Gpi8p is responsible for the recognition of the GPI signal sequence. Moreover, the unknown ORFs isolated by the multicopy suppressor screen described in chapter 3.4. should be analyzed in more detail and a synthetic lethality screen with the ts mutants *gpi8-2* or *gpi8-3* should be performed after further analysis of these mutants such as the dosage of the expression level of Gpi8p at the restrictive temperature. Mutant 3.1 although not yet proven to be a *gpi8*-linked mutant should also be included in this further analysis. After establishing the crystal structure of Gpi8p, sequencing of the dominant-negative mutants isolated in the screen for proteolytic *gpi8* alleles might be helpful to understand the precise functioning of Gpi8p. Finally, the relational database for yeast GPI protein has to be updated and evolved in order to remain a reliable tool for the analysis of the yeast GPI proteins and their GPI-SS sequence. ORFs which are candidates for remodelases are currently analyzed in the laboratory.

The identification of the active site in caspases and in the Gpi8p subfamily may also lead to the identification of the active site in C13 family members.

## 5. MATERIALS AND METHODS

Rarely used protocols and materials are specified in the Experimental Procedures or Methods Part of the corresponding Results chapter.

### *Strains, Media and Materials.*

*Saccharomyces cerevisiae* strains are listed in Table 1 and were grown in synthetic minimal medium supplemented with all 20 amino acids (20-400 mg/L), adenine sulfate, and uracile (SDaa medium). SGalaa medium is SDaa medium with 2 % galactose instead of glucose. The copper concentration of media was determined with the copper chelating agent BCS (Watkins *et al.*, 1971) and amounted to 0.75 – 1.0  $\mu$ M. Copper-free media contained 100  $\mu$ M BCS and 1  $\mu$ M FeCl<sub>2</sub>. 5-FOA containing media (1 mg/ml) contained 100 mg/L uracile. The absorbance of dilute cell suspensions was measured at 600 nm, one OD<sub>600</sub> unit of cells corresponded to 1-2.5 $\times 10^7$  cells as estimated by cell counting by means of a Neubauer chamber.

Reagents were purchased from the following sources: Bathocuproine-disulfonic acid disodium salt (BCS) from FLUKA; 5-fluoroorotic acid (FOA) from Toronto Research Chemicals Inc. (North York, Ontario, Canada); MG-132 from Biomol Research Laboratories (Plymouth, USA); [2-<sup>3</sup>H]-*myo*-inositol, 20 Ci/mmol from Anawa; [<sup>35</sup>S]-methionine/[<sup>35</sup>S]-cysteine Prot. Laboratory Mix from Moravек; X-OMAT films, zymolyase 20T, anti-mouse and anti-rabbit IgG-peroxidase conjugates from Sigma; anti-CRD antibodies from Oxford Glycosystems; anti-c-myc antibodies from BAbCO (Richmond, CA); glass beads (0.45-0.50 mm) from Braun Melsungen; TLC plates from MERCK; EN<sup>3</sup>HANCE spray and solution from NEN (Dupont); PCR primers were from Microsynth GmbH (Balgach, Switzerland); Taq DNA polymerase from Roche; bacteriophage T4 ligase from Pharmacia; restriction enzymes from NewEngland Biolabs; molecular biology kits from Macherey Nagel; antibodies against Cwp1, Galp,  $\alpha$ -galactosidase, and Yap3p were kindly donated by Dr. H. Shimoi (National Research Institute of Brewing, Kagamiyama, Japan), Dr. Howard Riezman (Biocenter, Basel, Switzerland), Dr. H. Kapteyn (Biocenter Amsterdam, Netherlands), and Dr. Y. Bourbonnais (University Laval, Québec, Canada), respectively. Plasmids pDH15 and pDH17, and strain RH392-3A were kindly provided by Dr. H. Riezman (Biocenter, Basel, Switzerland); plasmid pGGA1 from Dr. Marteen P. Scheurer (University of Amsterdam, Netherlands); plasmids pFBY166 from Dr. Jutta Heim (Novartis, Basel, Switzerland). Cassettes for producing knockouts of *DER3* and *UBC7* were a kind gift of Dr. D.H. Wolf (University of Stuttgart, Germany), and strain BK27 was kindly provided by Dr. M. Bard (University of Indianapolis, Indiana, USA). The production of anti-Galp (Fankhauser and Conzelmann, 1991), of anti-CPY (Meyer *et al.*, 2000) and of affinity purified anti-Gpi8p (Benghezal *et al.*, 1996) rabbit antibodies has been described previously.

### *Plasmids and Molecular Biology Protocols*

Plasmids are listed in Table 2 and were constructed using standard procedures (Sambrook *et al.*, 1989). Constructions are explained in the corresponding Result chapter.

***Growth curves***

Growth of liquid cultures was analyzed as described (Meyer *et al.*, 2000).

***Protein extraction and Western blot analysis***

Proteins were extracted by the glass beads lysis method (Meyer *et al.*, 2000) or by the rapid protein extraction protocol (Horvath and Riezman, 1994). After the addition of reducing sample buffer in different concentrations, extracts were denatured during 5' at 95 °C and separated by SDS-PAGE (Laemmli, 1970). The following gel concentrations were used for SDS-PAGE: for Gpi8p,  $\alpha$ -galactosidase and Cwp1p 10 %; for Gas1p and Yap3p 7.5 %; for CPY 8 and 9%. Western blots were developed by enhanced chemiluminescence (ECL Kit, Amersham, Buckinghamshire, UK). The following antibody concentrations were used: for anti-Gpi8p 1:3000; for anti-Gas1p 1:5'000-1:20'000; for anti-Cwp1p 1:1000; for anti- $\alpha$ -galactosidase 1:10'000, for anti-Yap3 1:2000; for anti-CRD and anti-Gaa1p 1:500; for anti-mouse and anti-rabbit IgG-peroxidase conjugates 1:2000. The expression of proteins was quantitated by densitometry using a Bio-Rad Imaging Densitometer, Model GS-700, and the Molecular Analyst 2.1 software (Bio-Rad Laboratories, Glattbrugg, Switzerland).

***Radiolabeling of GPI lipids and GPI proteins***

Cells were preincubated for the indicated time and labeled with [2-<sup>3</sup>H]-*myo*-inositol (2  $\mu$ Ci/OD<sub>600</sub> of cells) for the indicated time (generally 20') at 37 °C as described (Benghezal *et al.*, 1995). Lipids were extracted with chloroform/methanol/water 10:10:3 (v/v/v) and desalted by butanol/water phase separation as described (Sipos *et al.*, 1994). Lipid extracts were analyzed by ascending TLC using 0.2 mm-thick silica gel 60 plates with the solvent system chloroform/methanol/water 10:10:3. [2-<sup>3</sup>H]-*myo*-inositol and [2-<sup>3</sup>H]mannose-labeled standards were produced as described previously (Benghezal *et al.*, 1995). Radioactivity was detected by one- and two-dimensional radioscanning and fluorography. Maturation of Gas1p and CPY was analyzed by pulse-chase labeling experiments at 37 °C with [<sup>35</sup>S]-methionine/[<sup>35</sup>S]-cysteine (20  $\mu$ Ci/OD<sub>600</sub> of cells) followed by immunoprecipitation using polyclonal rabbit antisera and protein A-Sepharose as described previously (Horvath *et al.*, 1994; Meyer *et al.*, 2000).

Table 1. *S. cerevisiae* strains

strain	genotype	plasmid
W303-1A	<i>MATa ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15</i>	
W303-1B	<i>MATα ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15</i>	
X2180-1A	<i>MATa SUC2 mal gal2 CUP1</i>	
RH 392-3A	<i>MATa ura3-1 leu2-3,112 trp1-1 lys2 Δgas1::LEU2</i>	
HMSF176	<i>MATa sec18-1</i>	
BK27	<i>MATα leu2-3,112 lys9 ura3-52 Δerg6::LEU2</i>	
FBY11	<i>MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1</i>	
FBY122	<i>MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 gpi7-1 gpi8-1</i>	
FBY164	<i>MATa ade2-1 leu2-3,112 his3-11,15 Δgpi8::kanMX2</i> <i>ura3-1::URA3-GAL1-10-GPI8</i>	
FBY525	<i>MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 lys<sup>-</sup></i> <i>Δgpi8::kanMX2</i>	YEpGPI8
FBY526	FBY525	pBF53
FBY534	FBY525	pBF54
FBY535	FBY525	pBF55
FBY554	FBY525	pBF53_H54A <sup>a</sup>
FBY555	FBY525	pBF53_S60A <sup>a</sup>
FBY558	FBY525	pBF53_S60C <sup>a</sup>
FBY577	W303-1B; <i>ura3-1::URA3-GAL1-10-GPI8-myc-his6</i>	
FBY578	W303-1B; <i>ura3-1::URA3-GAL1-10-GPI8_C199A-myc-his6</i>	
FBY579	W303-1B; <i>ura3-1::URA3-GAL1-10-GPI8_S60→C59-myc-his6</i>	
FBY580 <sup>b</sup>	<i>leu2-3,112 ura<sup>-</sup> trp1-1 Δerg6::LEU2</i>	
FBY581 <sup>c</sup>	W303-1B; <i>Δder3</i>	
FBY582 <sup>c</sup>	W303-1B; <i>Δubc7</i>	
FBY143	<i>MATa/α ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 TRP1-1/trp1-1 lys<sup>+</sup>/lys<sup>-</sup> GPI8/Δgpi8::kanMX2</i>	

<sup>a</sup> For plasmids which harbour mutant alleles of *GPI8*, the type of mutation is indicated after the plasmid name. Rarely used strains are only described in the corresponding Results section.

<sup>b</sup> Strain FBY580 was obtained by crossing BK27 and W303-1A followed by tetrad dissection of diploids. Diploids were grown on SDaa medium lacking lysine and leucine and spores were then selected for growth on SDaa medium lacking leucine, tryptophane auxotrophy and a cold-sensitive growth phenotype. *Δerg6/trp1-1* cells are cold-sensitive for growth (Gaber *et al.*, 1989).

<sup>c</sup> Strains FBY581 and FBY582 were obtained by transforming W303-1B cells with the corresponding knock out cassette kindly provided by Dr. D. H. Wolf.

Table 2. Plasmids

plasmid	description	reference
YCplac22	<i>TRP1 CEN4-ARS1 Ap<sup>R</sup></i>	(Gietz and Sugino, 1988)
YIplac211	<i>URA3 Ap<sup>R</sup></i>	(Gietz and Sugino, 1988)
YIplac128	<i>LEU2 Ap<sup>R</sup></i>	(Gietz and Sugino, 1988)
YEplac112	<i>TRP1 2<math>\mu</math> Ap<sup>R</sup></i>	(Gietz and Sugino, 1988)
YEplac195	<i>URA3 2<math>\mu</math> Ap<sup>R</sup></i>	(Gietz and Sugino, 1988)
YEp352	<i>URA3 2<math>\mu</math> Ap<sup>R</sup></i>	(Hill <i>et al.</i> , 1986)
YEpGPI8	YEp352; <i>GPI8</i>	(Benghezal <i>et al.</i> , 1996)
YEpGPI8*	YEpGPI8; <i>gpi8</i> allele library	chapter 3.2.
YIpGAL	<i>URA3, Ap<sup>R</sup></i>	(te Heesen <i>et al.</i> , 1992)
YIpGALGPI8	YIpGAL; <i>GAL1-10-GPI8</i>	(Meyer <i>et al.</i> , 2000)
pBF51	YEp620; <i>GPI8</i>	(Barnes and Thorner, 1986)
pBF53	YCplac22; <i>GPI8</i>	(Meyer <i>et al.</i> , 2000)
pBF54	YCplac22; <i>CUP1-GPI8</i>	(Meyer <i>et al.</i> , 2000)
pBF55	YEplac112; <i>CUP1-GPI8</i>	(Meyer <i>et al.</i> , 2000)
pBF58	YEplac112; <i>GAL1-10-GPI8</i>	chapter 3.2.
pBF59	YIplac128; <i>GAL1-10-GPI8</i>	chapter 3.2.
pBF511	YIplac211; <i>GPI8</i>	chapter 3.5.
pBF515	YIplac211; <i>GPI8</i> (truncated <i>GPI8</i> promoter)	chapter 3.5.
pBF515*	pBF515; <i>gpi8</i> allele library (truncated <i>GPI8</i> promoter)	chapter 3.5.
pBF521	YCplac22; <i>GPI8</i> (truncated <i>GPI8</i> promoter)	chapter 3.5.
pBF522	pBF515; <i>GPI8-myc-his6</i> (truncated <i>GPI8</i> promoter)	chapter 3.5.
pCNYCG-C	<i>URA3; CEN-ARS; gas1</i> ( $\omega$ site mutant)	(Hamburger <i>et al.</i> , 1995)
pCNYCG-TN	<i>URA3; CEN-ARS; gas1</i> (spacer mutant)	(Hamburger <i>et al.</i> , 1995)
pDH15	YEplac195; <i>GAAI</i>	(Hamburger <i>et al.</i> , 1995)
pDH17	YEplac112; <i>GAAI</i>	(Hamburger <i>et al.</i> , 1995)

For plasmids which harbour mutant alleles of *GPI8*, the mutant description is added at the end of the name of the corresponding plasmid harboring the wt *GPI8* allele. Rarely used plasmids are only described in the corresponding Results section.

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## 7. WWW-SITES

### 7.1. Introduction

The world wide web (www) is an eldorado for life scientists. A wealth of different databases can be found which collect information on a broad range of organisms such as sequences and references; and several molecular biology servers with a variety of specialized software tools to analyze and compute the collected data can easily be accessed. In Table 1, www sites which I frequently addressed are listed with their category, uniform resource locator and name. In general, only homepages of different molecular biology servers are mentioned which give an overview on links to specialized sequence analysis software. However, the URL of very frequently accessed sites is directly specified. A nearly complete list of biology www sites can be found at "Amos Bairoch links for life scientists": URL: <http://www.expasy.ch/alinks.html>. (Last update: 03.08.2000)

### 7.2. List of www sites

**Table 1. List of www sites.**

Category and URL	Name
<i><b>Yeast genome and proteome</b></i>	
<a href="http://genome-www.stanford.edu/Saccharomyces/">http://genome-www.stanford.edu/Saccharomyces/</a>	<b>SGD</b> ( <i>Saccharomyces cerevisiae</i> Genome Database)
<a href="http://www.proteome.com/databases/index.html">http://www.proteome.com/databases/index.html</a>	<b>YPD</b> (Yeast Proteome Database)
<a href="http://www.mips.biochem.mpg.de/proj/yeast/">http://www.mips.biochem.mpg.de/proj/yeast/</a>	<b>MIPS</b> (Munich Information center for Protein Sequences)
<a href="http://genome-www.stanford.edu/Saccharomyces/chromosomes.html">http://genome-www.stanford.edu/Saccharomyces/chromosomes.html</a>	Saccharomyces cerevisiae systematic sequencing table
<i><b>Quick access to Yeast proteins and genes</b></i>	
<a href="http://www.proteome.com/databases/YPD/YPDsearch-long.html">http://www.proteome.com/databases/YPD/YPDsearch-long.html</a>	YPD full search form; search name or protein features
<a href="http://genome-www.stanford.edu/cgi-bin/SGD/search">http://genome-www.stanford.edu/cgi-bin/SGD/search</a>	SGD search form
<a href="http://speedy.mips.biochem.mpg.de/mips/programs/search_alias.html">http://speedy.mips.biochem.mpg.de/mips/programs/search_alias.html</a>	MIPS Yeast Code Search
<i><b>Other WWW sites for yeast resources are listed at:</b></i>	
<a href="http://genome-www.stanford.edu/Saccharomyces/yeast_info.html">http://genome-www.stanford.edu/Saccharomyces/yeast_info.html</a>	SGD Yeast WWW sites
<a href="http://www.expasy.ch/cgi-bin/lists-yeast?yeast.txt">http://www.expasy.ch/cgi-bin/lists-yeast?yeast.txt</a>	Links to S. cerevisiae WWW sites
<i><b>Yeast deletion strains</b></i>	
<a href="http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html">http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html</a>	<b>EUROSCARF</b> (European <i>Saccharomyces cerevisiae</i> archive for functional analysis)
<a href="http://www.resgen.com/">http://www.resgen.com/</a>	<b>Research Genetics</b>
<a href="http://sequence-">http://sequence-</a>	<b>Stanford yeast deletion</b>



<a href="http://www.stanford.edu/group/yeast_deletion_project/Enter_DB.html">www.stanford.edu/group/yeast_deletion_project/Enter_DB.html</a> <a href="http://www.atcc.org/L2.cfm?Lexp2=2&amp;Lexp3=2&amp;B=3&amp;Lexp4=14&amp;Lexp5=1">http://www.atcc.org/L2.cfm?Lexp2=2&amp;Lexp3=2&amp;B=3&amp;Lexp4=14&amp;Lexp5=1</a>	<b>database</b> <b>Yeast Genetic Stock Center at ATCC</b>
<b><i>Yeast primers and ORFs (DNA)</i></b> <a href="http://www.resgen.com/">http://www.resgen.com/</a>	<b>Research Genetics</b>
<b><i>DNA microarrays</i></b> <a href="http://cmgm.stanford.edu/pbrown/">http://cmgm.stanford.edu/pbrown/</a> <a href="http://industry.ebi.ac.uk/~alan/MicroArray/">http://industry.ebi.ac.uk/~alan/MicroArray/</a>	<b>The Brown Lab, Stanford University</b> <b>Large-Scale Gene Expression and Microarray Links and Resources</b>
<b><i>Yeast newsgroups/e-mail addresses:</i></b> <a href="http://www.bio.net/hypermail/YEAST/">http://www.bio.net/hypermail/YEAST/</a> <a href="http://www.gen.sbg.ac.at/projects/yeast/y~knots.html">http://www.gen.sbg.ac.at/projects/yeast/y~knots.html</a> <a href="http://www.bio.net/">http://www.bio.net/</a> <a href="http://www.molbio.princeton.edu/broach/newsgroups.html">http://www.molbio.princeton.edu/broach/newsgroups.html</a>	<b>YEAST bionet</b> <b>list of Yeast Bionets</b> <b>BIOSCI Newsgroup</b> <b>e-mail addresses of yeast researchers and yeast conference archives</b>
<b><i>Yeast functional analysis</i></b> <a href="http://websvr.mips.biochem.mpg.de/proj/eurofan/index.html">http://websvr.mips.biochem.mpg.de/proj/eurofan/index.html</a>	<b>MIPS EUROFAN</b> <b>Homepage</b>
<b><i>Miscellaneous Saccharomyces information sources</i></b> <a href="http://www.gen.sbg.ac.at/projects/yeast/y~knots.html">http://www.gen.sbg.ac.at/projects/yeast/y~knots.html</a>	
<b><i>General gene and protein sequences</i></b> <a href="http://www.expasy.ch/srs5/">http://www.expasy.ch/srs5/</a> <a href="http://www.expasy.ch/sprot/">http://www.expasy.ch/sprot/</a> <a href="http://www.expasy.ch/cgi-bin/sprot-search-de">http://www.expasy.ch/cgi-bin/sprot-search-de</a> <a href="http://www.bis.med.jhmi.edu/Dan/fields/pir.form.html">http://www.bis.med.jhmi.edu/Dan/fields/pir.form.html</a> <a href="http://www.mips.biochem.mpg.de/">http://www.mips.biochem.mpg.de/</a> <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a> <a href="http://www.ncbi.nlm.nih.gov/genbank/query_form.html">http://www.ncbi.nlm.nih.gov/genbank/query_form.html</a> <a href="http://www.ebi.ac.uk/embl/">http://www.ebi.ac.uk/embl/</a> <a href="http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html">http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html</a> <a href="http://www.ddbj.nig.ac.jp/">http://www.ddbj.nig.ac.jp/</a> <a href="http://flybase.bio.indiana.edu/">http://flybase.bio.indiana.edu/</a> <a href="http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml">http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml</a> <a href="http://genome-www.stanford.edu/Arabidopsis/">http://genome-www.stanford.edu/Arabidopsis/</a>	<b>Sequence Retrieval System</b> <b>SwissProt and TrEMBL</b> <b>Quick access to SwissProt or TrEMBL or both</b> <b>PIR (Protein Information Resource)</b> <b>Munich information center for protein sequences</b> <b>NCBI (National Center for Biotechnology information)</b> <b>Quick access to the Genbank database at NCBI</b> <b>EMBL nucleotide sequence database (EBI)</b> <b>NCBI Genbank searching</b> <b>DNA Data Bank of Japan</b> <b>FlyBase, a database of the Drosophila Genome</b> <b>AceDB, Caenorhabditis elegans genome database</b> <b>Arabidopsis thaliana genome database</b>

<p><b><i>General protein sequence analysis</i></b></p> <p><a href="http://www.expasy.ch/">http://www.expasy.ch/</a>  <a href="http://cbrg.inf.ethz.ch/ServerBooklet/ServerBooklet.html">http://cbrg.inf.ethz.ch/ServerBooklet/ServerBooklet.html</a></p> <p><a href="http://www.infobiosud.univ-montp1.fr/SERVEUR/PROPSEARCH/propsearch.html">http://www.infobiosud.univ-montp1.fr/SERVEUR/PROPSEARCH/propsearch.html</a></p> <p><a href="http://psort.nibb.ac.jp:8800">http://psort.nibb.ac.jp:8800</a></p> <p><a href="http://www.public.iastate.edu/~pedro/rt_1.html">http://www.public.iastate.edu/~pedro/rt_1.html</a></p> <p><a href="http://www.expasy.ch/tools/peptide-mass.html">http://www.expasy.ch/tools/peptide-mass.html</a></p>	<p><b>ExPASy</b>  The computational Biochemistry server at ETHZ  <b>PROPSEARCH</b> (Protein family identification from protein features predicted from the primary sequence)  <b>PSORT</b> (Prediction of Protein Sorting Signals and localization sites)  Pedro's Molecular Biology search and analysis  <b>PeptideMass</b> (molecular weight of peptides)</p>
<p><b><i>Sequence comparison</i></b></p> <p><a href="http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd">http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd</a>  <a href="http://www.ch.embnet.org/software/frameBLAST.html?">http://www.ch.embnet.org/software/frameBLAST.html?</a></p> <p><a href="http://www.ncbi.nlm.nih.gov/BLAST/">http://www.ncbi.nlm.nih.gov/BLAST/</a></p> <p><a href="http://protein.toulouse.inra.fr/prodom/doc/blast_form.html">http://protein.toulouse.inra.fr/prodom/doc/blast_form.html</a>  <a href="http://cbrg.inf.ethz.ch/subsection3_1_2.html#SECTION000120000000000000">http://cbrg.inf.ethz.ch/subsection3_1_2.html#SECTION000120000000000000</a></p>	<p><b>BLAST2.0 at SGD</b>  <b>BLAST2.0 at SIB</b> (Swiss Institute of Bioinformatics) different <b>BLAST programs at NCBI</b>  <b>BLAST at ProDom</b>  Search several sequences against SwissProt</p>
<p><b><i>Multiple sequence alignments</i></b></p> <p><a href="http://www2.ebi.ac.uk/clustalw/">http://www2.ebi.ac.uk/clustalw/</a>  <a href="http://www.bork.embl-heidelberg.de/Alignment/">http://www.bork.embl-heidelberg.de/Alignment/</a></p> <p><a href="http://www.blocks.fhcrc.org/process_blocks.html">http://www.blocks.fhcrc.org/process_blocks.html</a></p> <p><a href="http://www.ch.embnet.org/software/BOX_form.html">http://www.ch.embnet.org/software/BOX_form.html</a></p>	<p><b>Clustalw at EBI</b>  Multiple sequence alignment  <b>Blocks Multiple Alignment Processor</b>  <b>BOXSHADE 3.21</b> (Pretty Printing and Shading of Multiple-Alignment files)</p>
<p><b><i>Patterns, motifs, domains and profiles</i></b></p> <p><a href="http://www.expasy.ch/prosite/">http://www.expasy.ch/prosite/</a></p> <p><a href="http://www2.ebi.ac.uk/pratt/">http://www2.ebi.ac.uk/pratt/</a></p> <p><a href="http://www.expasy.ch/tools/scnpsit2.html">http://www.expasy.ch/tools/scnpsit2.html</a></p> <p><a href="http://motif.stanford.edu/projects.html">http://motif.stanford.edu/projects.html</a></p> <p><a href="http://www.blocks.fhcrc.org/blockmkr/make_blocks.html">http://www.blocks.fhcrc.org/blockmkr/make_blocks.html</a>  <a href="http://exon.stanford.edu/cgi-bin/profile/get-block.pl/">http://exon.stanford.edu/cgi-bin/profile/get-block.pl/</a></p> <p><a href="http://protein.toulouse.inra.fr/prodom/doc/prodom.html">http://protein.toulouse.inra.fr/prodom/doc/prodom.html</a></p> <p><a href="http://www.isrec.isb-sib.ch/profile/profile.html">http://www.isrec.isb-sib.ch/profile/profile.html</a></p>	<p>search <b>PROSITE</b> (database of protein families and domains)  <b>Pratt at EBI</b> (Protein pattern or fingerprint discovery)  pattern against SwissProt and TrEMBL  <b>emotif</b> (make, search and scan motifs)  <b>Block Maker</b>  selection of defined motifs (blocks)  <b>ProDom</b> (the protein domain database)  <b>ISREC Profile Homepage</b></p>

<a href="http://www.ch.embnet.org/software/PFSCAN_form.html">http://www.ch.embnet.org/software/PFSCAN_form.html</a> <a href="http://pfam.wustl.edu/">http://pfam.wustl.edu/</a>	<b>ISREC ProfileScan Server</b> <b>Pfam</b> (protein family database)
<i>Phylogenetic trees/evolution genetics</i>	
<a href="http://evolution.genetics.washington.edu/phylip/software.html">http://evolution.genetics.washington.edu/phylip/software.html</a> <a href="http://taxonomy.zoology.gla.ac.uk/rod/treeview/treeview_manual.html#Toc356614768">http://taxonomy.zoology.gla.ac.uk/rod/treeview/treeview_manual.html#Toc356614768</a> <a href="http://evolution.genetics.washington.edu/phylip.html">http://evolution.genetics.washington.edu/phylip.html</a> <a href="http://alt-www.uia.ac.be/u/yvdp/treecon.html">http://alt-www.uia.ac.be/u/yvdp/treecon.html</a> <a href="http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html">http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html</a>	phylogeny programs <b>TreeView</b>  <b>PHYLIP</b> <b>TREECON</b> The NCBI taxonomy homepage
<i>Protein 3D structure</i>	
<a href="http://www.rcsb.org/pdb/">http://www.rcsb.org/pdb/</a> <a href="http://www.expasy.ch/sw3d/">http://www.expasy.ch/sw3d/</a>	Protein Data Bank <b>Swiss 3D image</b> , Database of annotated 3D image
<i>Protein 2D-PAGE</i>	
<a href="http://www.expasy.ch/ch2d/2d-index.html">http://www.expasy.ch/ch2d/2d-index.html</a>  <a href="http://yeast-2dpage.gmm.gu.se/">http://yeast-2dpage.gmm.gu.se/</a> <a href="http://www.ibgc.u-bordeaux2.fr/YPM/accueil.htm">http://www.ibgc.u-bordeaux2.fr/YPM/accueil.htm</a>	<b>WORLD-2DPAGE</b> Index to 2-D PAGE databases and services Yeast 2-D page Yeast Protein Map, 2-D gel map of <i>Saccharomyces cerevisiae</i> proteins
<i>Oligonucleotides and sequencing</i>	
<a href="http://www.microsynth.ch/">http://www.microsynth.ch/</a>	Microsynth GmbH
<i>Bioinformatics/Molecular Biology servers</i>	
<a href="http://www.isb-sib.ch/">http://www.isb-sib.ch/</a>  <a href="http://motif.stanford.edu/">http://motif.stanford.edu/</a>  <a href="http://www.ebi.ac.uk/">http://www.ebi.ac.uk/</a>  <a href="http://pbil.univ-lyon1.fr/">http://pbil.univ-lyon1.fr/</a>  <a href="http://www.expasy.ch/">http://www.expasy.ch/</a> <a href="http://www.ch.embnet.org/">http://www.ch.embnet.org/</a> <a href="http://www.bork.embl-heidelberg.de/">http://www.bork.embl-heidelberg.de/</a>  <a href="http://blocks.fhcrc.org/blocks/">http://blocks.fhcrc.org/blocks/</a>  <a href="http://www.expasy.ch/alinks.html">http://www.expasy.ch/alinks.html</a>  <a href="http://www.public.iastate.edu/~pedro/research_tools.html">http://www.public.iastate.edu/~pedro/research_tools.html</a>  <a href="http://ictsg10.unil.ch:8080/gcgusers/umeyer/">http://ictsg10.unil.ch:8080/gcgusers/umeyer/</a>	<b>Swiss Institute of Bioinformatics</b> The Brutlag Bioinformatics Group, Stanford University <b>EBI</b> (European Bioinformatics Institute) <b>PBIL</b> (Pôle Bio-Informatique Lyonnais) <b>ExPASy</b> Swiss node of EMBnet Bork group at MDC, Berlin <b>Blocks WWW Server</b>  <b>Amos Bairoch's links for life scientists with an interest in biological macromolecules</b> Pedro's Molecular Biology research tools GCG account at ISREC (institut de chimie thérapeutique silicon)

	graphics)
<i>Vectors</i>	
<a href="http://vectordb.atcg.com/">http://vectordb.atcg.com/</a> <a href="http://www.geocities.com/HotSprings/Spa/3013/plasmid.html">http://www.geocities.com/HotSprings/Spa/3013/plasmid.html</a>	Vector database Plasmid Data Bank
<i>Searching literature</i>	
<a href="http://www.ncbi.nlm.nih.gov/PubMed/">http://www.ncbi.nlm.nih.gov/PubMed/</a>  <a href="http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&amp;form=0">http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&amp;form=0</a> <a href="http://www.medportal.com/medline.html">http://www.medportal.com/medline.html</a> <a href="http://www.biomednet.com/db/medline/search">http://www.biomednet.com/db/medline/search</a> <a href="http://www.docnet.org.uk/DRFELIX/">http://www.docnet.org.uk/DRFELIX/</a>  <a href="http://www.ethbib.ethz.ch/ejour/ejour.html">http://www.ethbib.ethz.ch/ejour/ejour.html</a>  <a href="http://www.amazon.com/exec/obidos/subst/home/home.html/002-7967725-7481451">http://www.amazon.com/exec/obidos/subst/home/home.html/002-7967725-7481451</a>	<b>PubMed</b> (search tool for accessing biomedical literature citations) Direct access to Medline at NCBI The Medline Fool BioMedNet Medline Dr Felix's Free MEDLINE® Page Library at ETH (Electronically available journals) Amazon books
<i>Biochemical journals</i>	
<a href="http://www.expasy.ch/cgi-bin/jourlist?jourlist.txt">http://www.expasy.ch/cgi-bin/jourlist?jourlist.txt</a>  <a href="http://www.ncbi.nlm.nih.gov/PubMed/fulltext.html">http://www.ncbi.nlm.nih.gov/PubMed/fulltext.html</a>  <a href="http://www.cell.com/">http://www.cell.com/</a> <a href="http://www.nature.com/">http://www.nature.com/</a> <a href="http://www.sciencemag.org/">http://www.sciencemag.org/</a> <a href="http://www.jcb.org/">http://www.jcb.org/</a>  <a href="http://www.emboj.org/">http://www.emboj.org/</a> <a href="http://www.jbc.org/">http://www.jbc.org/</a>  <a href="http://www.pnas.org/">http://www.pnas.org/</a> <a href="http://mcb.asm.org/">http://mcb.asm.org/</a>  <a href="http://www.molbiolcell.org/">http://www.molbiolcell.org/</a>  <a href="http://www.academicpress.com/jmb">http://www.academicpress.com/jmb</a>  <a href="http://www.blacksci.co.uk/products/journals/ejb.htm">http://www.blacksci.co.uk/products/journals/ejb.htm</a>  <a href="http://www.portlandpress.co.uk/bj/">http://www.portlandpress.co.uk/bj/</a>  <a href="http://JB.bcasj.or.jp/">http://JB.bcasj.or.jp/</a>  <a href="http://www.cob.org.uk/JCS/">http://www.cob.org.uk/JCS/</a>  <a href="http://www.elsevier.nl/locate/inca/506085">http://www.elsevier.nl/locate/inca/506085</a>  <a href="http://www.elsevier.nl/locate/inca/506033">http://www.elsevier.nl/locate/inca/506033</a>	<b>SwissProt journals list</b> (Provides direct links to most biological journals home pages) MEDLINE Journals With Links to Publisher Web Sites Direct access to Cell Direct access to Nature Direct access to Science Direct access to J. Cell Biol. Direct access to EMBO J. Direct access to J. Biol. Chem. Direct access to PNAS Direct access to Mol. Cell Biol. Direct access to Mol. Biol. Cell Direct access to J. Mol. Biol. Direct access to Europ. J. Biochem. Direct access to Biochem. J. Direct access to J. Biochem. Direct access to J. Cell Sci. Direct access to FEBS Lett. Direct access to Gene

<a href="http://www.genetics.org/">http://www.genetics.org/</a> <a href="http://jb.asm.org/">http://jb.asm.org/</a>  <a href="http://www.academicpress.com/mie">http://www.academicpress.com/mie</a>  <a href="http://www.biomednet.com/library/tibs">http://www.biomednet.com/library/tibs</a> <a href="http://acsinfo.acs.org/journals/bichaw/">http://acsinfo.acs.org/journals/bichaw/</a>	Direct access to Genetics Direct access to J. Bacteriol. Direct access to Meth. Enzymol. Direct access to TiBS Direct access to Biochemistry
<i>Genomics</i> <a href="http://www.gene.ucl.ac.uk/hugo/">http://www.gene.ucl.ac.uk/hugo/</a>	<b>Human Genome Organisation</b>
<i>Strains and DNA sources</i> <a href="http://www.atcc.org/">http://www.atcc.org/</a>  <a href="http://www.resgen.com/">http://www.resgen.com/</a> <a href="http://www.biosyn.com/">http://www.biosyn.com/</a> <a href="http://www.sciquest.com/">http://www.sciquest.com/</a>	<b>ATCC</b> (American Type Culture Collection) Research Genetics Bio-Synthesis Incorporated Sciquest.com (The internet source for scientific products)
<i>E-mail addresses</i> <a href="gopher://gopher.gdb.org/11/biol-search/yeast-email">gopher://gopher.gdb.org/11/biol-search/yeast-email</a>  <a href="http://www.bigfoot.com/">http://www.bigfoot.com/</a>	e-mail addresses of yeast researchers e-mail and white page searches
<i>GPI proteins</i> <a href="http://www.mad-cow.org/~tom/gpi.html">http://www.mad-cow.org/~tom/gpi.html</a> <a href="http://www.embl-heidelberg.de/~beisenha/GPI/gpi.html">http://www.embl-heidelberg.de/~beisenha/GPI/gpi.html</a>  <a href="http://www.biozentrum.unibas.ch/~biocomp/report/5th/riezmann.htm">http://www.biozentrum.unibas.ch/~biocomp/report/5th/riezmann.htm</a> <a href="http://mendel.imp.univie.ac.at/gpi/gpi_server.html">http://mendel.imp.univie.ac.at/gpi/gpi_server.html</a>	GPI overview <b>Sequence analysis of GPI-anchored proteins on the proprotein level</b> Membrane traffic in yeast  <b>big-PI Predictor</b> (predicts omega site of metazoan and protazoan GPI proteins)
<i>Micellaneous</i> <a href="http://www.snf.ch/">http://www.snf.ch/</a>  <a href="http://sgich1.unifr.ch/bc/bc.html">http://sgich1.unifr.ch/bc/bc.html</a>	Swiss National Science Foundation Institute of Biochemistry, University of Fribourg
<i>search engines</i> <a href="http://www.cyber411.com/main.htm">http://www.cyber411.com/main.htm</a>  <a href="http://www.go2net.com/search.html">http://www.go2net.com/search.html</a>  <a href="http://www.amdahl.com/internet/meta-index.html">http://www.amdahl.com/internet/meta-index.html</a> <a href="http://www.altavista.com/">http://www.altavista.com/</a> <a href="http://www.yahoo.com/">http://www.yahoo.com/</a> <a href="http://www.hotbot.com/">http://www.hotbot.com/</a> <a href="http://infoseek.go.com/">http://infoseek.go.com/</a>	cyber411 (combined search with 16 search engines) metacrawler (combined search with 10 search engines) search engine index AltaVista yahoo HotBot InfoSeek

<a href="http://www.google.com/">http://www.google.com/</a>	Google
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Sites frequently used for the remodelase screen (Chapter 3.6.1) are highlighted in bold  
Please refer to references as cited on the www sites.

## **8. ANNEX**

### **8.1. Candidates for GPI lipid remodelases (selection step 2)**

## Y

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		YLR390w.....	177		



**Complete search results obtained by BLAST searches (Part I).** Proteins denoted unknown in the databases MIPS and YPD (January 1998).

1. First priority proteins:

- **YJL132W**: Chrs. X; protein: S55180 (750 aas)
  - weak similarity to human PLD
  - homologous to human GPI-PLDs
  - homologous to bovine GPI-PLD
  - shares the putative motif DVSWHSL with the two human GPI-PLDs (this motif has been found in two other possible *S. cerevisiae* proteins which are both unknown: S53585 (YAL06 (PIR), 126 aas); P32613 (YEF8\_YEAST (SwissProt), 152 aas)); this motif is also present in human PLD; in bovine GPI-PLD you find DVNWHSL
  - it is not a member of any protein family or homology domain group
  - putative transmembrane protein (1 TM domain predicted)
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): outside>vacuole>peroxisome>ER
- **YDR018C**: Chrs. IV; protein: S54641 (396 aas)
  - homologous to 1-acyl-sn-glycerol-3-phosphate acyltransferase (*Cocos nucifera*)
  - weakly homologous to Slc1p
  - strong similarity to hypothetical protein YBR042C (cf. second priority proteins)
  - it is not a member of any protein family or homology domain group
  - putative transmembrane protein (3 TM domains predicted); most likely a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>), favored for ER or Golgi localization
  - seems to have no N-terminal signal sequence, but this protein has a KKXX motif
  - predicted localization (PSORT): plasma membrane>Golgi>ER or peroxisome
- **YNL101W**: Chrs. XIV; protein: Z71377 (713 aas)
  - weak homology to Faa4p and to Are1p
  - similar to members of a putative transporter family (YBR089\*, YER064\*, YER119\*, YIL088\*, YKL146\*, YNL101\*)  
no further information has been found about the possible function of these gene products (not found as homologs of lipid-binding enzymes)
  - it is not a member of any protein family or homology domain group
  - putative transmembrane protein (9 TM domains predicted); most likely a Type IIIa membrane protein (N<sub>cyt</sub> C<sub>exo</sub>).
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): plasma membrane>Golgi>ER or peroxisome
- **YLR020C** Chrs. XII; protein: Z73192x1 (538 aas)
  - similar to triacylglycerol lipase (Tgl1p)
  - putative transmembrane protein (1 TM domain predicted); seems to be a Type Ib membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
  - seems to have an uncleavable N-terminal signal sequence; eventually intramitochondrial signal sequence  
predicted localization (PSORT): ER (mb) >> perox. > PM > ER (luminal)
- **YLL048C** Chrs. XII; protein: Z73153x1 (1661 aas), identified: vacuolar protease
  - similar to Pat1p and Pat2p
  - homologous to putative transport proteins YHL035C and YKR103W
  - putative ABC transporter, has an ATP/GTP-binding site motif A (P-loop)
  - putative transmembrane protein (12 TM domains predicted); seems to be a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
  - seems to have no N-terminal signal sequence; C terminus is KKD (?)  
predicted localization (PSORT): PM > Golgi > ER or perox.
- **YDR452W**: Chrs. IV; protein: U33007\*37 (674 aas)
  - similar to human acid sphingomyelinase (produces ceramide and phosphocholine, PLC-like) and sphingomyelin phosphodiesterase (human and *M. musculus*, PLD-like?)
  - it is not a member of any protein family or homology domain group
  - putative transmembrane protein (1 TM domain predicted); seems to be a Type II membrane protein (N<sub>cyt</sub> C<sub>exo</sub>)
  - seems to have no N-terminal signal sequence

- predicted localization (PSORT): mitochondrial inner membrane>plasma membrane>nucleus>Golgi
- **YHR080C**: Chrs. VIII; protein: P3880 (1345 aas),
- homologous to Slc1p-derived domain consensus sequence
  - similar to hypothetical proteins YDR326C (transmembrane), YFL042C (no TM) and YLR072W (transmembrane)
  - ATP/GTP binding site  
putative transmembrane protein (1 TM domain predicted);
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus>peroxisome>mitochondrial matrix>ER

## 2. Second priority proteins:

- **YNR008W**: Chrs. XIV; protein: Z71623 (661 aas)
  - similar to PC:sterol O-acyltransferase precursor (human and *Oryctolagus cuniculus*)
  - unknown
  - putative transmembrane protein (1 TM domain predicted); seems to be a Type II (N<sub>cyt</sub> C<sub>exo</sub>)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): plasma membrane > peroxisome > Golgi > nucleus
- **YDR434W**: Chrs. IV; protein: S69714 (950 aas)
  - weak similarity to PI-PLC (rat mRNA)
  - it is not a member of any protein family or homology domain group
  - putative transmembrane protein (2 TM domains predicted) ); seems to be a Type IIIa (N<sub>cyt</sub> C<sub>exo</sub>)
  - seems to have an uncleavable N-terminal signal sequence; C terminus: EDEL (ER retrieval, anyway it is most probably not soluble)
  - predicted localization (PSORT): ER (mb) > PM > Golgi > ER (lumenal)
- **YNR074C**: Chrs. XIV; protein: Z71689 (378 aas)
  - weak similarity to acyl-CoA synthetase (*S. cerevisiae*)
  - homologous to NAD(P)H binding proteins
  - homologous to nitrite reductase (*B. subtilis*)
  - unknown
  - putative transmembrane protein (1 TM domain predicted) ); seems to be a Type II membrane protein (N<sub>cyt</sub> C<sub>exo</sub>, length of cytoplasmic tail: 7 aas)
  - seems to have an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): PM > ER (mb) > Golgi > ER (lumenal)
- **YBR042C**: Chrs. II; protein: S45900 (397 aas)
  - similar to 1-acyl-glycerol-3-phosphate acyltransferase (*Brassica napus*) and YDR018C (cf. first priority proteins)
  - probable membrane-bound small GTPase
  - unknown
  - putative transmembrane protein (4 TM domains predicted); seems to be a Type IIIa membrane protein (N<sub>cyt</sub> C<sub>exo</sub>)
  - seems to have an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): ER(mb) > PM > Golgi > ER (lumenal)
- **YMR006C** Chrs. XIII; protein: Z48613 (706 aas); identified: PLB2
  - putative *lyso*-phospholipase (EUROFAN node lipid metabolism)
  - strong similarity to PIB1p (63.9 % identity in 664 aa overlap)
  - has similarity to Spo1p (transcription regulator, *spo1* is sporulation defective)
  - putative transmembrane protein (1 TM domain predicted); seems to be a Type Ia membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM > ER (membrane) > ER (lumenal) > outside
- **YLL012W** Chrs. XII; protein: Z73117x1 (573 aas)
  - similar to triacylglycerol lipase (Tgl1p)
  - putative transmembrane protein (2 TM domains predicted)
  - seems to have a cleavable N-terminal signal sequence; eventually intramitochondrial signal sequence
  - predicted localization (PSORT): outside > perox. > ER (membrane or lumenal)
- **YLL015W** Chrs. XII; protein: Z73120x1 (1559 aas)
  - similar to Pat1p and Pat2p
  - putative ABC transporter, has similarity to metal resistance proteins, has an ATP/GTP-binding site motif A (P-loop)
  - putative transmembrane protein (10 TM domains predicted); seems to be a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
  - seems to have no or an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): PM > Golgi > ER, perox.
- **YHL035C** Chrs. VIII; protein: P38735 (1592 aas)
  - similar to Pat1p and Pat2p

- putative ABC transporter, has similarity to multidrug resistance proteins, has an ATP/GTP-binding site motif A (P-loop)
- putative transmembrane protein (12 TM domains predicted); seems to be a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM > Golgi > ER or perox.

- YKR103W Chrs. XI; protein: P36028 (1218 aas)

- similar to Pat1p and Pat2p
- similar to Yhl035p, Ycf1p (metal resistance protein with similarity to human cystic fibrosis protein CFTR and multidrug resistance proteins, major localization is the vacuole) and to CFTR protein
- EGF-like domain cysteine pattern signature
- putative ABC transporter, has similarity to multidrug resistance proteins, has an ATP/GTP-binding site motif A (P-loop)
- putative transmembrane protein (13 TM domains predicted); seems to be a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM > Golgi > ER or perox.

3. Third priority proteins:

- YKR018C: Chrs. XI; protein: S38087 (725 aas)

- weak similarity to human acid sphingomyelinase
- highly similar to YJL082 (67% identity over 725 aas; not found as lipid binding protein, 731 aas, 1 TM domain predicted, unknown)
- it is not a member of any protein family or homology domain group
- putative transmembrane protein (1 TM domain predicted); seems to be a Type Ib membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM > ER (mb) > mitoch. inner membrane

- YML023C: Chrs. XIII; protein: S49754 (556 aas)

- weak similarity to sphingomyelin phosphodiesterase (*M. musculus*)
- it is not a member of any protein family or homology domain group
- putative transmembrane protein (2 TM domains predicted) ); seems to be a Type IIIa membrane protein (N<sub>cyt</sub> C<sub>exo</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM > Golgi > ER

- YPR042C Chrs. XVI; protein: Z73616x2 (1075 aas)

- similar to Cat2p
- similarity to Jsn1p (43 % identity over 1027 aas; *jsn1* is a benomyl dependent tubulin mutant)
- rich in asparagine near the C-terminus
- putative transmembrane protein (1 TM domain predicted); seems to be a Type II membrane protein (N<sub>cyt</sub> C<sub>exo</sub>)
- seems to have no cleavable N-terminal signal sequence
- predicted localization (PSORT): ER >> PM > perox. > mitoch. inner membrane

- YOR161C Chrs. XV; protein: Z75069x1 (539 aas)

- weakly homologous to Slc1p-derived domain consensus sequence
- putative transmembrane protein (8 to 11 TM domains predicted); seems to be a Type IIIb membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM > Golgi > ER, perox.

- YOL075C Chrs. XV; protein: Z74817x1 (1095 aas)

- similar to Pat2p
- similarity to *Anopheles gambiae* ATP-binding cassette (ABC) protein
- putative ABC transporter
- may be separated from YOL074C by a frameshift (no TM domains predicted, YOL075C includes the ORF YOL075C)

- putative transmembrane protein (7 TM domains predicted); seems to be a Type IIIa membrane protein (N<sub>cyt</sub> C<sub>exo</sub>)
- seems to have no N-terminal signal sequence
- predicted localization (PSORT): PM > Golgi > ER or perox.

#### 4. Miscellaneous results:

putative lipid-binding GPI-anchored proteins:

- YOL011W Chrs. XV; protein: Z74753x1 (686 aas)

- putative phospholipase (EUROFAN node lipid metabolism), strong similarity to phospholipases
- putative transmembrane protein (2 TM domains predicted); seems to be a Type Ia membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have a cleavable N-terminal signal sequence and to be GPI-anchored
- predicted localization (PSORT): PM >> microbody > ER

- YIL011W Chrs. IX; protein: Z47047x167 (269 aas); identified: PLB4

- similar to PIB1p, serine-rich protein (Tip1p/Tir1p family) ), stress-induced proteins SRP1/TIR1 family signature
- putative transmembrane protein (2 TM domains predicted); seems to be a Type Ia membrane protein (N<sub>exo</sub> C<sub>cyt</sub>)
- seems to have a cleavable N-terminal signal sequence and to be GPI-anchored
- predicted localization (PSORT): PM >> ER > outside

**Complete search results obtained by homology searches (PartII).** Proteins denoted unknown in the databases MIPS and YPD (January 1998).

1. acyltransferases:

- **YPR140w**: Chrs. XVI; (381 aas), 814386-815528
  - found with acyltransferase/YDR018c profile (Kay)
  - similar to human Barth syndrome gene taffazin
  - no TM domain predicted, but has a KKKXX motif
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): peroxisome>mitoch. matrix
- **YBL011w**: Chrs. XIII; (759 aas); SCT1, 203495-105771
  - found with acyltransferase/YDR018c profile (Kay)
  - suppressor of the choline transport mutant *ctrl*
  - 3 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>Golgi, ER
- **YKR067w**: Chrs. XI; (743 aas), 567557-569785
  - found with acyltransferase/YDR018c profile (Kay Hoffmann)
  - similar to Sct1p
  - 5 TM domains predicted; Type IIIb (Nexo Ccyt) which is favored for ER mb; has KKX at the very C terminus (?)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>Golgi, ER
- cf. also YBR042c, YDR018c (first part)

2. Aur1p/PA phosphatase:

- **YJL134W**: Chrs. X; (409 aas); LCB3
  - found with Aur1p/PA phosphatase profile (Kay)
  - involved in incorporation of exogenous long chain bases in sphingolipids; KO is viable and has a reduced rate of incorporation of long chain base into sphingolipids, but YKR053 (to which LCB3 is similar) null mutant does not; KO is more resistant to growth inhibition by long chain bases; KO is more resistant to canavanine and hygromycin B; *lcb3/ybr053c* double null mutations have no synergistic effect; putative GPI anchor attachment site; putative transporter of sphingoid long chain bases
  - 4(-8) TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>Golgi, mitoch. IM, ER
- **YKR053c**: Chrs. XI; (404 aas), 534921-533710
  - found with Aur1p/PA phosphatase profile (Kay)
  - similar to Lcb3p; KO is viable; not redundant with Lcb3p (cf. YJL134w); KO has the same sensitivity to growth inhibition by long chain bases, canavanine and hygromycin B as wild-type cells; putative GPI anchor attachment site;
  - 5 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>Golgi, ER
- **YGR036c**: Chrs. VII; (239 aas), CWH8, 558862-558146
  - identical to CWH8, Calcofluor White hypersensitive
  - found with Aur1p/PA phosphatase profile (Kay)
  - contains 3 stretches of amino acids that are characteristic for a wide variety of phosphatases, including lipid phosphatases and a protein phosphatase
  - ko viable, severely affected growth rate, hypo-N-glycosylation of secretory proteins, severely reduced levels of dolichol-linked oligosaccharides
  - similar to *Treponema denticola* phosphatase
  - 4 TM domains predicted; Type IIIb (Nexo Ccyt) which is favored for ER mb; has a KKKXX motif
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER

- **YDR503c**: Chrs. IV; (274 aas); 1455869-1455048
  - found with Aur1p/PA phosphatase profile (Kay)
  - related to YDR284c (DAG pyrophosphate phosphatase)
  - similarity to unknown *C. elegans* protein 8U28738\*5
  - 5 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): ER, PM>Golgi

### 3. neutral SMase:

- **YER019W**: Chrs. V; (477 aas); 192796-194226
  - found as homolog of mammalian nSMase (Kay) which cuts sphingomyelin and also PC (remaining activity: 30 %)
  - has typical ATP/GTP-binding sites
  - KO is viable
  - 2 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>Golgi, ER

cf. also YDR452w similar to human acid sphingomyelinase (cutting only SM)

### 4. lipase/esterase/thioesterase:

- **YFL025c**: Chrs. VI; (1029 aas); BST1 87232-84146
  - has the PROSITE motif PDOC00110 (PS50187) at position 136-251 [LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-G-[GSTAC] with the serine as active site
  - 10 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM>mitoch., Golgi
  - BLAST (NCBI): has unknown homologs
- **YOR022c**: Chrs. XV; (715 aas); 375856-373712
  - has the PROSITE motif PDOC00110 (PS50187) at position 494-516
  - has no TM domain, but a KKKXX motif
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): mitoch.
  - BLAST (NCBI): mouse membrane-bound associated PI transfer protein, probable calcium transporter
- **YOR059c**: Chrs. XV; (450 aas); 440259-438910
  - has the PROSITE motif PDOC00110 (PS50187) at position 6-105
  - P loop (ATP/GTP)
  - 1 TM domain predicted; Type IIa (Ncyt Cexo) which is favored for ER mb when the relative position of the cytoplasmic tail is bigger than 30 % (here 59 %); has HKXX at the very C terminus (?)
  - seems to have an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): ER>PM, Golgi
  - BLAST (NCBI): has unknown homologs (YGL144c, YDL109c, etc.)
- **YBR204c**: Chrs. II; (375 aas); 633335-632211
  - has the PROSITE motif PDOC00110 (PS50187) at position 87-192
  - similar to peroxisomal serine-active site lipase
  - 1 TM domain predicted; Type Ib (Nexo Ccyt)
  - has a C-terminal SLK peroxisomal targeting signal
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): Perox. > ER
  - BLAST (NCBI): putative triacylglycerol lipase 2 in *Mycoplasma pneumoniae*

cf. also YNR008w (lipase; first part)

## 5. GPI10 homologs:

- **YNR030w**: Chrs. XIV; (551 aas); ECM39 678796-680448
  - homologous to GPI10; involved in cell wall structure and biosynthesis; Greek key motif signature found in beta and gamma crystallins
  - 7 TM domains predicted; Type IIIa which is favored for ER mb
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
  - BLAST (NCBI): homologous to PIG-B, GPI10 and unknown proteins, weak similarity to Smp3p (YOR149c)
- **YOR149c**: Chrs. XV; (516 aas); SMP3, 611386-609839
  - homologous to GPI10; required for plasmid maintenance, functions in the protein kinase C pathway
  - KO is lethal
  - 6 TM domains predicted; Type IIIa
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
  - BLAST (NCBI): homologous to PIG-B, GPI10 and unknown proteins, weak similarity to Smp3p (YOR149c)

## 6. ECM mutants with predicted TM domains (cf. also YBL101c/ECM21): Tn3 insertion into the corresponding genes causes hypersensitivity to the calcofluor white, these genes are therefore assumed to be involved in cell wall structure and biosynthesis (Lussier et al., Genetics 147, 435 (1997)):

- **YOR092w**: (613 aas); ECM3 495125-496963 (gene locus)
  - strongly similar to YNL095p
  - 8 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, mitochond. IM, Golgi, ER
  - BLAST (NCBI): homologous to unknown proteins
- **YML048w**: Chrs. XIII; (403 aas); ECM6, EFF2 178426-179634
  - plays a role in glucose repression, synergistic effect with Gsf1p on SUC2 suppression
  - 1 TM domain predicted; Type II (Ncyt Cexo); has a KKXX motif
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): ER
  - BLAST (NCBI): homologous to unknown proteins
- **YLR443w**: Chrs. XII; (448 aas); ECM7 1022619-1023962
  - 4 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): mitochond. or PM
  - BLAST (NCBI): homologous to unknown proteins
- **YKR004c**: Chrs. XI; (292 aas); ECM9 447318-446443 (gene locus)
  - 1 TM domain predicted
  - hypersensitive to the glycolipid papulacandin, thought to be an inhibitor of beta-1,3-glucan synthesis
  - has introns
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): Cyt, perox.
  - BLAST (NCBI): appears to be for the moment unique since no homologs were found
- **YHR021w-a**: Chrs. XIII, (151 aas); ECM12 149216-149668
  - similar to YNL095p
  - questionable ORF
  - seems to have an uncleavable N-terminal signal sequence
  - 1 TM domains predicted; Type II Ncyt Cexo
  - PSORT: PM, ER
- **YMR128w**: Chrs. XIII ( aas); ECM16 523695-527495
  - similar to ATP-dependent RNA helicases of the dead/death-box family; P loop (ATP/GTP-binding motif A)
  - 1 TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus> perox.



- **YJR106w**: Chrs. X (725 aas); ECM27 624525-626699
  - 12 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - weak similarity to acylglycerol lipase YJR107w
  - predicted localization (PSORT): PM, Golgi, ER
  - BLAST (NCBI): homologous to unknown protein in *C. elegans*, similar to sodium/calcium potassium exchanging protein (antiporter)
- **YHL030w**: Chrs. VIII (1868 aas); ECM29 40082-45685
  - 2 TM domains predicted; Type IIIa (Ncyt Cexo) which is favored for ER mb
  - ko viable
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
  - BLAST (NCBI): homologous to unknown proteins
- **YLR436c**: Chrs. XII; (1274 aas); ECM30 1011239-1007418
  - has a phosphopantetheine attachment site
  - 2 TM domains predicted; Type IIIb (Nexo Ccyt) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, mitoch. IM, ER
  - BLAST (NCBI): no homologs found
- **YER176w**: Chrs. V (1121 aas); ECM32 HEL1 identified: DNA dependent ATPase/DNA helicase B 541686-545040
  - similar to DNA helicases Dna2p and Nam7p
  - KO is viable
  - 1 TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus, perox.
  - BLAST (NCBI): homologous to Sen1p (helicase) and unknown proteins
- **YHL043w**: Chrs. VIII (170 aas); ECM34 14899-15408
  - similar to subtelomerically-encoded proteins (such as the cos genes)
  - 2 TM domains predicted; Type IIIb (Nexo Ccyt) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus, PM, Golgi, ER
  - BLAST (NCBI): homologous to quite a lot of unknown proteins
- **YOL025w**: Chrs. XV (680 aas); ECM36/LAG2
  - protein involved in longevity
  - 1 TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): mitoch.
  - BLAST (NCBI): no homologs
- **YIL146c**: Chrs. IX (529 aas); ECM37 75773-74187
  - 1 TM domains predicted; Type II (Ncyt Cexo); cytoplasmic tail from 1-394 (74 %, favors ER mb)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus or ER
  - BLAST (NCBI): no homologs
- **YNR030w**: Chrs. XIV (551 aas); ECM39 **Hegemann**
  - cf. GPI10 homologs
- **YOR030w**: (619 aas); ECM41/DFG16 386824-388680
  - protein involved in invasive growth upon nitrogen starvation
  - 7 TM domains predicted; Type IIIb(Nexo Ccyt) which is favored for ER mb
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, mitoch. IM, ER
  - BLAST (NCBI): no homologs found
- **YCL007c**: Chrs. III, (130 aas); ECM36
  - protein that affects the mannoprotein layer of the cell wall

- similar to human  $\alpha$ -glucosidase
  - 1 TM domain predicted; Type II (Ncyt Cexo)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): ER, nucleus, PM, perox.
- YLR353w: Chrs. XII, (603 aas); BUD8
- protein required for bipolar budding, has an RNA recognition (RRM) domain
  - mutant is hypersensitive to CFW and zymolase, resistant to caffeine (inhibitor of cAMP phosphodiesterase), abnormal budding pattern in diploid cells, but axial budding in haploid cells is normal
  - 2 TM domains predicted; Type IIIb (Nexo Ccyt)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- YBR078w: Chrs. II, (468 aas); ECM33
- hypersensitive to CFW, zymolase, hygromycin, killer toxin, decreased level of mannose to glucose in the cell wall
  - 2 TM domains predicted; likely type IIIa
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- YAL059w: Chrs. I, (212 aas); ECM1
- probably a TMD protein
  - seems to have an uncleavable N-terminal signal sequence
  - predicted localization (PSORT): ER

Proteins without TM domains:

- **YBL001c**: Chrs. II, (104 aas); ECM15
  - mutant sensitive to CFW and hygromycin B
  - similar to *Staphylococcus xylosus* glucose kinase
  - 0 TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): cytoplasm, but has an KKXXX C terminal signal sequence
- **YLR390w**: Chrs. XII, (112 aas); ECM19
  - has about 14 mRNA copies per cell
  - Lussier TMD protein
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus, mitoch.
- **YPR011c**: Chrs. XVI; (326 aas); 584037-583060
  - similar to GPI-PLD; contains PS00215 (mitoch. energy transfer proteins); homologous to human Grave's disease carrier protein, similarity to ATP/ADP carrier proteins
  - no TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): Cyt, perox.
- **YJL068c**: Chrs. X; (299 aas)
  - has the PROSITE motif PDOC00110 (lipase)
  - no TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): Perox. > Cyt.
  - BLAST (NCBI): similar to human esterase D (40 %)
- **YNL040w**: Chrs. XIV; (450 aas)
  - has phospholipase A2 active sites signature (PLA2-His)
  - no TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): ?
- **YER024w**: Chrs. V; (923 aas); 202191-204959
  - has the acyltransferase C2 pattern PS00440 (acyltransferases ChoActase, COT, CPT family signature 2)
  - KO is viable
  - no TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus > perox. > mitoch.
  - BLAST (NCBI): homologous to mouse, human, rat, yeast mitochondrial carnitine palmitoyltransferase, choline O-acetyltransferase
- other unknown proteins having the PROSITE motif PDOC00110 (lipase) and without a predicted TM domain:
  - YJR107w
  - YDL109c (similar to YGL144c), strong similarity to thiamine repressed protein
  - YLR099c (motif at position 71-198, mitochondrial energy transfer proteins signature, homologous to acetyltransferase *C. elegans*, atropinesterase, prolinase, carboxylesterase, YDR125c, Ecm18p) YDR125c 702751-701393
  - YDR444w 1350279-1352339 similar to YDL109c and YGL144c
  - YPR147c 826554-825643
- miscellaneous (without TM):
  - YKR020w Chrs. XI 477979-478470; (164 aas): weakly similar to a "forced" GPI-PLD/GPI10 consensus; seems to have no N-terminal signal sequence; Cyt or mitoch. matrix
  - YKL061w Chrs. XI; (113) 325416-325754: weakly similar to a "forced" GPI10/GPI3 consensus; seems to have no N-terminal signal sequence; Cyt or mitoch. matrix
  - YMR073c Chrs. XIII, (201) 41872-412270; similar to Scs7p and to rat cytochrome B5 (heme binding domain signature)

## 8. ORFs identified by Klis (personal communication), CFW hypersensitive:

- **YBR255w**: Chrs. II; (694 aas)
  - unknown function and no related genes found (YPD)
  - 2 TM domains predicted; Type IIIb (Nexo Ccyt)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): nucleus, PM, Golgi, ER
- **YBR266c**: Chrs. II; (150 aas)
  - unknown function and no related genes found
  - 2 TM domains predicted; Type IIIa (Ncyt Cexo)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): mitoch. or PM
- **YDL074c**: Chrs. IV; (700 aas)
  - unknown function and no related genes found
  - 1 TM domain predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): cytoplasm or perox.
- **YJL062w**: Chrs. X; (830 aas)
  - unknown function and member of the major facilitator superfamily (MFS) which includes Mcd4p, Yjl062p, Yil031p and other.
  - 11 TM domains predicted; Type IIIa (Ncyt Cexo)
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- **YJL070c**: Chrs. X; (888 aas)
  - similarity to rat AMP deaminase 1
  - 1 TM domains predicted
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): perox., mitoch., ER
- **YJL094c**: Chrs. X; (873 aas)
  - similarity to enterococcus hirae Na<sup>+</sup>/H<sup>+</sup> antiporter NAPA
  - one of the three putative Na<sup>+</sup>/H<sup>+</sup> antiporters in yeast
  - member of the major facilitator superfamily
  - ko viable
  - 13 TM domains predicted; Type IIIb (Nexo Ccyt)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- **YNL159c**: Chrs. XIV; (289 aas)
  - unknown function and no related genes
  - 3 TM domains predicted; Type IIIa Ncyt Cexo
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- **YBR162c**: Chrs. II; (455 aas)
  - similar to Aga1p, Yil171p, Tir2p, about 10 mRNA copies per cell
  - ko viable
  - 1 TM domains predicted
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): outside, ER, Golgi
- **YDR015c**: Chrs. IV; 129 aas)
  - unknown function and no related genes, evtl. a transporter
  - 1 TM domains predicted; Type II Ncyt Cexo
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, perox., Golgi, ER
- **YJR001w**: Chrs. X; (602 aas)
  - member of the major facilitator superfamily

- 9 TM domains predicted; Type IIIb Nexo Ccyt
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- YNL058c: Chrs. XIV; (316 aas)
- similarity to Yil117p, has a putative PEST motif
  - 1 or 2 TM domains predicted; Type Ia
  - seems to have a cleavable N-terminal signal sequence
  - predicted localization (PSORT): PM, (ER, outside)
- YNL095c: Chrs. XIV; (642 aas)
- strongly similar to Ecm3p
  - 10 TM domains predicted; Type IIIa Ncyt Cexo
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER, mitoch.
- YNL294c: Chrs. XIV; (533 aas)
- unknown function and no related genes
  - 6 TM domains predicted; Type IIIb Nexo Ccyt
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER
- YOL152w: Chrs. XV; (629 aas) FRE7
- similar Frp1 of *S. pombe* and related to 8 other yeast proteins (Fre1p-Fre6p, Ygl160p, Ylr047p) which may comprise a gene family of transmembrane electron transporters
  - has potential flavine adenine nucleotide binding domain
  - 7 TM domains predicted; Type IIIa (Ncyt Cexo)
  - seems to have no N-terminal signal sequence
  - predicted localization (PSORT): PM, Golgi, ER, perox.

9. ORFs identified by Bankaitis (personal communication):

- YNL325c: Chrs. XIV; (879 aas) FIG4
- suppressor of *sac1* mutation and protein induced by mating factor
  - similar to Sac1p, Inp51p and Inp52p (both inositol polyphosphate-5-phosphatases)
  - 0 TM domains predicted
  - predicted localization (PSORT):
- YOR109w: Chrs. XIV; (1107 aas) INP53
- inositol polyphosphate-5-phosphatase
  - synaptojanin-like protein (nerve terminal protein with inositol-5-phosphatase activity)
  - mutant is defective in Golgi-endosome traffic, suppresses *pma1-7* ts growth defect, abnormal vacuolar morphology
  - ko viable
  - involved in the endosomal pathway
  - 1 TM domains predicted
  - predicted localization (PSORT):
  - contains two conserved motifs: GDXN(Y/F)R and P(S/A)W(C/T)DRI

## 8.2. List of *S. cerevisiae* GPI anchored proteins with the predicted residues at the positions $\omega$ , $\omega+1$ and $\omega+2$

**Table 4. All 70 GPI proteins present in the Yeast GPI protein database.**

ORF	Name	$\omega$	$\omega+1$	$\omega+2$
YAL063c	Flo9	G	S	A
YAR050w	Flo1	G	S	A
YBR067c	Tip1	N	A	G
YBR078w	Ecm33	S	S	L
YCL048w		N	S	A
YCR061w		G	A	Q
YCR089w	Fig2	G	S	A
YCR098c	Git1	N	G	T
YDR055w		N	A	A
YDR077w	Sed1	N	G	A
YDR134c		S	P	A
YDR144c	Mkc7	N	G	G
YDR261c	Exg2	N	A	A
YDR349c	Yps7	G	A	G
YDR522c	Sps2	N	S	A
YDR534c		G	A	A
YEL040w	Utr2	N	A	G
YER011w	Tir1	G	A	A
YER150w	Spi1	N	A	A
YFL067w		G	I	S
YFR054c		S	S	L
YGL259w	Yps5	S	A	L
YGR014w	Msb2	S	S	S
YGR189c		N	G	A
YHR126c		G	A	G
YHR211w	Flo5	G	S	A
YIL011w	Yib1	N	A	G
YIR019c	Muc1	G	A	A
YIR039c	Yps6	N	A	G
YJL078c	Pry3	G	A	A
YJL171c		N	G	V
YJR004c	Sag1	G	K	A
YJR150c	Dan1	N	G	A
YJR151C		N	G	A
YKL046c		G	A	G
YKL096w	Cwp1	N	A	G
YKL097w	Cwp2	N	G	A
YKR102w	Flo10	G	I	A
YLR040c		N	G	A
YLR042c		G	V	G
YLR046c		D	K	S
YLR110c		G	A	A
YLR120c	Yap3	N	V	G
YLR121c	Yps3	N	S	A
YLR194c		N	G	A
YLR343w	Gas2	N	D	G

YLR391w-a	Ssr1	N	V	L
YMR006c	Plb2	N	A	A
YMR008c	Plb1	N	A	G
YMR200w	Rot1	N	T	S
YMR215w	Gas3	N	V	S
YMR307w	Gas1	N	A	A
YNL190w		N	A	A
YNL300w		G	A	A
YNL322c	Kre1	N	E	A
YNL327w	Egt2	G	A	A
YNR044w	Aga1	G	S	G
YOL011w	Plb3	S	H	L
YOL030w	Gas5	G	A	A
YOL132w	Gas4	N	S	A
YOL155c		G	I	A
YOR009w		N	G	A
YOR010c	Tir2	N	G	A
YOR146w		S	S	S
YOR214c		N	S	S
YOR382w		G	A	A
YOR383c		G	A	A
YPL130w	Spo19	N	A	S
YPL261c		N	D	A
	Sta1	A	F	N

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## DECLARATION FOR THE FACULTY

Urs Meyer  
Im Ried  
CH-3212 Gurmels

Fribourg, le 11 Octobre 2000

Aux personnes concernées

Sujet: Thèse présentée à l'Université de Fribourg (Suisse) pour l'obtention du grade de Docteur rerum naturalium.

Messieurs,

Par la présente, je certifie que j'ai rédigé ma thèse "*GPI anchor attachment in yeast: analysis of Gpi8p, the putative catalytic subunit of the GPI:protein transamidase*" moi-même et sur la base d'un travail personnel sans aide illicite.

U. Meyer

Urs Meyer

## CURRICULUM VITAE

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<b>DATE AND PLACE OF BIRTH</b>	28 <sup>th</sup> January 1972, Fribourg (Switzerland)
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### EDUCATION

1978 - 1984	Primary School, Gurmels
1984 - 1987	Secondary School, Gurmels
1987 - 1991	Collège Sainte-Croix, Fribourg
	Maturity diploma Type B (latin, scientific and linguistic)
1991 - 1995	Undergraduate degree in Biochemistry, Institute of Biochemistry, Faculty of Sciences, University of Fribourg, Switzerland
1996 -	PhD thesis in Biochemistry, Institute of Biochemistry, University of Fribourg, Switzerland. Supervisor: Prof. Dr A. Conzelmann.

### POSITIONS

1993	Research assistant at the Paul Scherrer Institute, Division of Radiopharmacy, Würenlingen und Villigen, Switzerland. Production of novel rhenium-186 compounds for anti-cancer radioimmunotherapy.
1994 -	Teaching assistant of lab courses for students of biochemistry, biology, pharmacy and medicine.



**TEACHING ACTIVITIES**

1997	Examinator of yeast genetics course
1996 -	Director of Diploma thesis of Markus Britschgi and Serge Summermatter

**ADDITIONAL TRAINING**

1995	Summer academy course. Swiss Foundation for Studies, Fund for gifted young people. Maienfeld, Switzerland.
	Combinatorial Methods in Chemistry.
1995-1999	III <sup>e</sup> Cycle Romand courses in Biological Sciences (Switzerland): <ul style="list-style-type: none"> <li>- Viruses as tools in research and therapy. Eurotel, Villars-sur-Ollon (1996).</li> <li>- Microtechniques in protein and peptide chemistry. microsequencing from 2D-gel spots. CMU, Geneva (1996).</li> <li>- Computer-based sequence analysis. Unix, GCG and internet resources programs. EPFL, Lausanne (1997).</li> <li>- Biological defense mechanisms. Eurotel, Villars-sur-Ollon (1997).</li> <li>- Computer-based sequence analysis. Effective use of the data originating from large-scale sequencing projects. EPFL, Lausanne (1998).</li> <li>- Introduction to computer modelling: 3D-structure of proteins. ISREC, Lausanne (1999)</li> <li>- DNA array workshop, ISREC, Lausanne (1999)</li> </ul>
1997 – 1998	Courses for the foundation of an own company. Business plan, marketing, accounting und code numbers. ETH tools, ETH, Zurich, Switzerland.
1998	Professional communication skills for academic researchers: writing skills for researchers. English Language Institute, University of Fribourg, Switzerland.
since 1999	Postgraduate studies on information systems. Institute of Informatics, University of Fribourg, Switzerland.

**SEMINARS AND CONFERENCES**

1995	Annual USBEB conference. Fribourg, Switzerland.
1996	Perspectives in Cell Biology. August 2 and 3, University of Basel, Basel, Switzerland.
1996	Annual USGEB conference. Zurich, Switzerland.
1997	Yeast lipid biosynthesis, remodelling and transport. May 9 and 10, Institute of Biochemistry, University of Fribourg, Fribourg, Switzerland.
1997	GPI anchors in biological membranes. September 14 – 17, Splügen, Switzerland.
1997	Annual USGEB conference. Geneva, Switzerland.

- 1997 Swiss Yeast Meeting. September 26, University of Berne, Berne, Switzerland.
- 1998 Poster presentation at the MDC symposium on Protein Transport and Stability. September 5 – 9, Berlin, Germany.
- 1998 Oral and poster presentation at the Swiss Yeast Meeting. October 9, University of Fribourg, Fribourg, Switzerland.
- 1998 Annual USGEB conference. Lausanne, Switzerland.
- 1999 FEBS'99 meeting, June 19-24 Nice, France

## PUBLICATIONS

### Scientific papers

Meyer,U., Benghezal,M., Imhof,I., and Conzelmann,A. (2000). Active site determination of Gpi8p, a caspase-related enzyme required for glycosylphosphatidylinositol anchor addition to proteins. *Biochemistry* 39, 3461-3471.

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Imhof,I., Canivenc-Gansel,E., Meyer,U., and Conzelmann,A. (2000) Phosphatidylethanolamine is the donor of the phosphorylethanolamine linked to the  $\alpha$ 1,4-linked mannose of yeast GPI structures. *Glycobiology*. In press.

Fraering, P., Imhof, I., Meyer, U., and Conzelmann, A. (2000) Functional and structural analysis of yeast Gpi8p, an essential component of the yeast GPI transamidase complex. submitted.

### Public relations

Votations fédérales du 7 juin 1998. Initiative pour la protection génétique. Conférence, 28 mai 1998.

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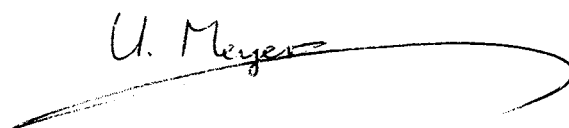
## MEMBERSHIPS

- since 1996 Swiss Society of Experimental Biology (USGEB).  
since 1996 Swiss Society of Biochemistry (SGB).

### PLACE AND DATE

Fribourg, 11.10.2000

### SIGNATURE

A handwritten signature in black ink, appearing to read 'U. Meyer', followed by a long, sweeping horizontal flourish.