Active Site Determination of Gpi8p, a Caspase-Related Enzyme Required for Glycosylphosphatidylinositol Anchor Addition to Proteins†

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Received September 21, 1999; Revised Manuscript Received December 3, 1999

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 Δ*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 Δ*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[1] 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

† This work was supported by a grant no. 3100-049664.96 from the Swiss National Foundation for Scientific Research.

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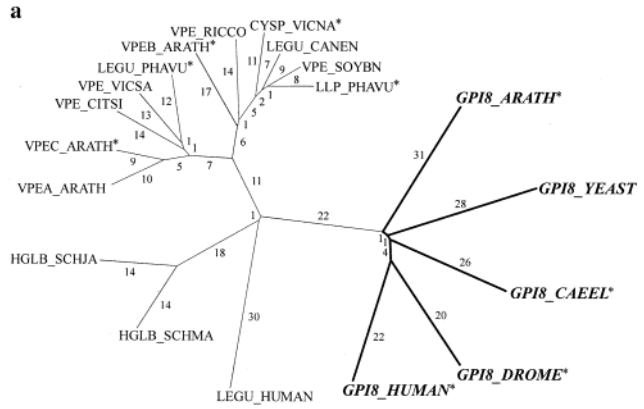
[1] Abbreviations: BCS, bathocuproinedisulfonic acid; CP, complete precursor; FOA, 5-fluoroorotic acid; GPI, glycosylphosphatidylinositol; Ins, *myo*-inositol; wt, wild-type.

10.1021/bi992186o CCC: $19.00
Published on Web 03/03/2000

a



b

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yGpi8  MRIAMHLPLLLLYIFLLPLSGANNTDAAHEVIATNTNNWAVLVSTSRFWF  50
Gpi8f            l    l          s   e    hTNNWAVLV tSrF
C13f             v                   p        rWavlvAGS Gy
                 :                            W::::  S

yGpi8  NYRHMANVLSMYRTVKRLGIPDSQIILMLSDDVACNSRNLFPGSVFNNKD 100
Gpi8f   y  v n Ls  rsvKRLGIPDs IilMladDmaCN RN  Pg Vf
C13f   NYRHQaD CHAyq l   G   EnIvvfMYDDIA    NP  G iiN
        :   :    : :   G    :I:::: :D::    N   : ::

yGpi8  HAIDLYGDSVEVDYRGYEVTVENFIRLLTDRWTEDHPKSKRLLTDENSNI 150
Gpi8f    nlYGddVEVDYRg EVTVEnF RvLTgR     prSKrLLsD  Sni
C13f     dvY G V  DY G  V   nf  vl Gd     gGGKVi S  nD i
         : Y   V  DY :  V   ::   :          K : :     :

yGpi8  FIYMTGHGGDDFLKFQDAEEIASEDIADAFQQMYEKKRYNEIFFMIDTCQ 200
Gpi8f  liYmTGHGGd FlKFQDsEEi s dlA a q Mwek RynEl fm DtCq
C13f   Fiy sDHGgpGvl  P    lya  f dvlk       Y  mViYvEAcE
        ::   HG:   :       : :  :   ::       :  : :  ::::

yGpi8  ANTMYSKFYSPNILAVGSSEMDESSYSHHSDVEIGVAVIDRFTYYCLDFL 250
Gpi8f  a smye f Spnvla  SS   E S Shh D  IGV viDRyTyY l Fl
C13f   SGSif   l  niYaTTAsna E Swg y         t LgD YS  Wm
       : :::      :: :  ::   E S   :              Y   ::

yGpi8  EQIDKNSTLTLQDLFDSFTFEKIHSHVGVRTDLFDRNPSEVLITDFFANV 300
Gpi8f       s     d f        S    RtD y  d   V iTdFFg
C13f   DS  hn  etlkqQY  vk  T  SHV qyGd  i    l  y G  pa
              :: : :         S     :
```

c

Position	-9	-8	-7	-6	-5	-4	-3	-2	-1	*	+1	+2
Caspase	K	P	K	LIVMF	LIVMF	LIVMF	LIVMF	Q	A	C	RQG	G
Gpi8f	KNH	E	ILM	LMF	IVF	IM	IVA	D	ST	C199	RQ	GAS
C13f	KSR	x	LM	V	IF	Y	LIV	E	A	CN	E	S

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_*CAEEL* 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 PSO1122).

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 μM. Copper-free media contained 100 μM BCS and 1 μM $FeCl_2$. The absorbance of dilute cell suspensions was measured at 600 nm, one OD_{600} unit of cells corresponding to $1-2.5 \times 10^7$ cells. Alternatively, cells were counted microscopically. Reagents were purchased from the following sources: Bathocuproinedisulfonic acid disodium salt (BCS) from Fluka; [2-^{3}H]-*myo*-inositol, 20 Ci/mmol from Anawa; [^{35}S]-methionine/[^{35}S]-cysteine Prot. Laboratory Mix, Moravek; 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. Gaa1p-

Table 1: Complementation of the Growth Defect of Δ*gpi8*[a]

transfected *gpi8* allele	tetrad analysis: total of tetrads	number of viable spores/tetrad: 0	1	2	3	4	plasmid shuffling: clones growing on FOA(%)
empty vector	9	1	1	7			0
GPI8	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

[a] FBY143 (Δ*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 *Nco*I. For plasmid shuffling (last column), the haploid strain FBY525 (Δ*gpi8*/YEp*GPI8*) 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 α-galactosidase/α-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 *yGPI8* 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 (CAAGCTGATGTCTGTCACGCT; S60→C59). To express mutant alleles from a single copy vector, the 2374 bp *Sst*I/*Sal*I fragment of YEp*GPI8*, harboring the complete *GPI8* gene, was introduced into the *Sst*I/*Sal*I sites of YCplac22, resulting in plasmid pBF53. Mutant alleles were generated in this vector by excising the 2286 bp *Xho*I/*Xba*I fragment of pBF53 and replacing it with the 2286 bp *Xho*I/*Xba*I fragments of YEp*GPI8* plasmids containing mutant alleles.

To generate *gpi8* alleles under the control of the *GAL1-10* promoter, a *Bam*HI restriction site was introduced by PCR upstream of the ATG of GPI8 by using the primers *GPI8*-for*Bam* (5′-AAGCCG*GGATCC*ATGCGTATAGCG*ATG*-3′) and *GPI8*-rev400 (5′-AAAAGTCTCGAGATTAGTGTACAGGTC-3′). The PCR fragment was digested with *Bam*HI and *Sal*I; the resulting 79 bp fragment was cloned together with the 1629 bp *Alw*NI/*Sal*I fragment of YEp*GPI8* into the vector YIpGal (*30*) digested with *Sst*I and *Xho*I to yield the plasmid YIp*GALGPI8*. Before transfection YIp*GALGPI8* was digested with *Stu*I in order to direct its integration into the *ura3-1* locus of W303-1B or FBY143. After transformation of FBY143 with YIp*GALGPI8*, ura$^+$ transformants were sporulated and tetrads were dissected and germinated on rich galactose medium. Δ*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 YIp*GALGPI8* with *Eco*RI and *Nci*I, 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 *Sal*I. Ligation of the 3752 fragment of pFBY166 and the 1712 bp fragment of YIp*GALGPI8* produced the plasmid pBF58. The 2335 bp *Sph*I/*Sal*I *CUP1*−*GPI8* fragment of pBF58 was then introduced into the *Sph*I/*Sal*I 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 μg/mL; 20 μL per OD_{600} 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-^{3}H]*myo*-inositol (2 μCi/OD_{600} 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-^{3}H]-*myo*-inositol- and [2-^{3}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 [^{35}S]methionine and [^{35}S]-

Table 2

(A) Strains Used in this Study

strain	genotype	plasmid
W303-1B	*MATα ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*	
FBY11	*MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1*	
FBY122	*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 gpi7-1 gpi8-1*	
FBY164	*MATa ade2-1 leu2-3,112 his3-11,15 Δgpi8::kanMX2 ura3-1::URA3-GAL1-10-GPI8*	
FBY525	*MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 lys⁻ Δgpi8::kanMX2*	YEp*GPI8*
FBY526	FBY525	pBF53
FBY577	W303-1B; *ura3-1::URA3-GAL1-10-GPI8-myc-his6*	
FBY578	W303-1B; *ura3-1::URA3-GAL1-10-GPI8*_C199A-*myc-his6*	
FBY554	FBY525	pBF53_H54A[a]
FBY555	FBY525	pBF53_S60A[a]
FBY558	FBY525	pBF53_S60C[a]
FBY143	*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⁺/lys-GPI8/Δgpi*8::kanMX2	

(B) Plasmids Used in This Study

plasmid	description	reference
YCplac22	*TRP1 CEN4-ARS1 Ap^R*	(*55*)
YEplac112	*TRP1 2μ Ap^R*	(*55*)
YEplac195	*URA3 2μ Ap^R*	(*55*)
YEp352	*URA3 2μ Ap^R*	(*56*)
YEp*GPI8*	YEp352; *GPI8*	(*28*)
YIp*GAL*	*URA3 Ap^R*	(*30*)
YIp*GALGPI8*	YIpGAL; *GAL1-10-GPI8*	this study
pBF53	YCplac22; *GPI8*	this study
pBF54	YCplac22; *CUP1-GPI8*	this study
pBF55	YEplac112; *CUP1-GPI8*	this study
pDH15	YEplac195; *GAA1*	(*9*)
pDH17	YEplac112; *GAA1*	(*9*)

[a] For plasmids which harbor mutant alleles of GPI8, the type of mutation is indicated after the plasmid name.

cysteine (20 μCi/OD_{600}) 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 Δgpi8 and gpi7-gpi8. Mutant alleles of *GPI8* were expressed in a Δ*gpi8/GPI8* heterozygote diploid strain, and the viability of the Δ*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 Δ*gpi8* colonies. Mutations C199A and H157A as well as S60→C59 were completely unable to rescue the Δ*gpi8* spores. H54A, S60A, and S60C were still able to complement Δ*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→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 Δ*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 Δ*gpi8* background using plasmid shuffling gave the same result as dissection of tetrads: C199A, H157A, and S60→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^{2+} inducible *CUP1* promoter. After transfection into wt cells and induction for 1 h with 500 μM Cu^{2+} 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-

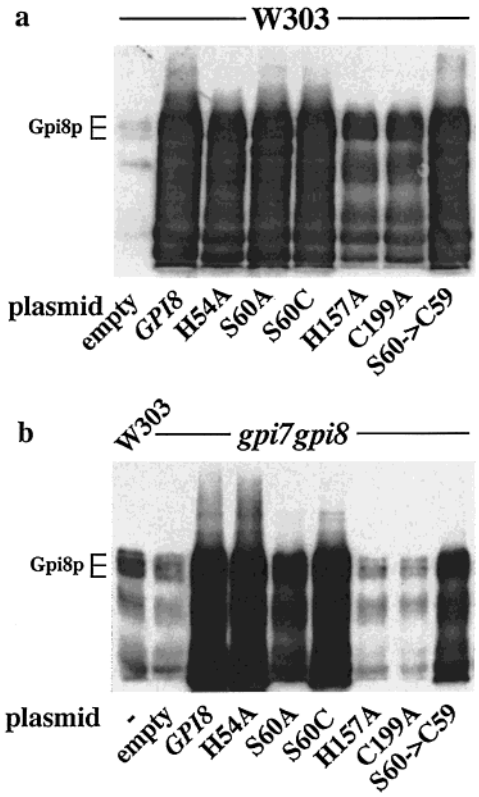

FIGURE 2: Overexpression of mutant Gpi8p in W303 and *gpi7−gpi8*. *gpi8* alleles were expressed under the control of the strong, copper inducible *CUP1* promoter using the multicopy vector pBF55 (Table 2B). Cells were cultured at 24 °C in SDaa (panel a) or copper-free SDaa/BCS (panel b) to exponential phase. One hour before extraction, conditions were changed in that cells were shifted to 37 °C (panel a) and that $CuSO_4$ was added to 500 μM (panel a) or 200 μM (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 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 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 *GAL1-10* 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 Cu^{2+} was suppressed by the concomitant overexpression of wt Gpi8p under the GAL1-10 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 μM Cu^{2+} nor on the kinetics by which cells harboring a *CUP1* promoter driven C199A ceased to grow upon addition of Cu^{2+} (not shown). Similarly, the growth arrest caused by the induction of *GAL1-10* 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 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 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}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 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}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 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

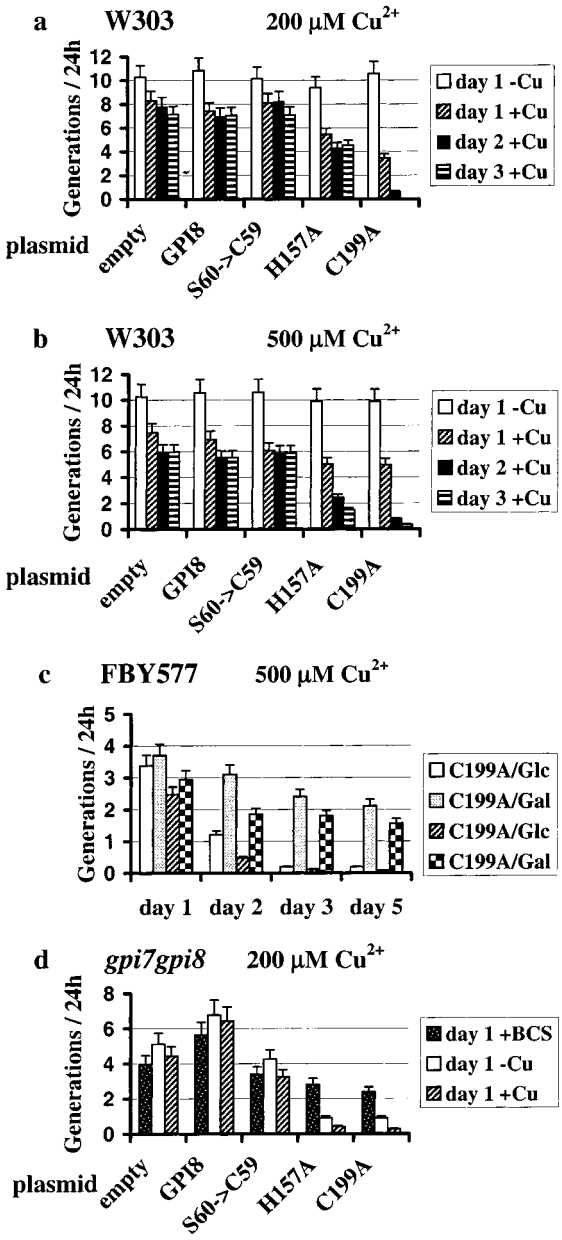

FIGURE 3: Expression of nonfunctional *gpi8* alleles H157A and C199A arrests cell growth of W303 and *gpi7*−*gpi8*. *gpi8* alleles under the control of the *CUP1* promoter were present on the multicopy or centromeric vectors pBF55 or pBF54, which were retained by omission of trp from growth media. Panels a, b: W303 cells, harboring *gpi8* alleles on pBF55, were grown to exponential phase at 24 °C without Cu^{2+} (0.75−1 μM Cu^{2+}) in SDaa medium and were resuspended at 0.2−0.3 OD_{600}/mL in fresh normal (−Cu) or $CuSO_4$-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 *GAL1-10* promoter) and harboring C199A under the *CUP1* promoter on pBF54 (light-shaded, dark-shaded) or pBF55 (slanted line, checkered) were resuspended at 0.2−0.3 OD_{600}/mL in fresh glucose or galactose medium and kept at 37 °C for 6 h. Cu^{2+} was then added to 500 μM 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 μM BCS) and then cultured further either in copper-free (BCS), normal (−Cu) or $CuSO_4$-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_{600}/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.

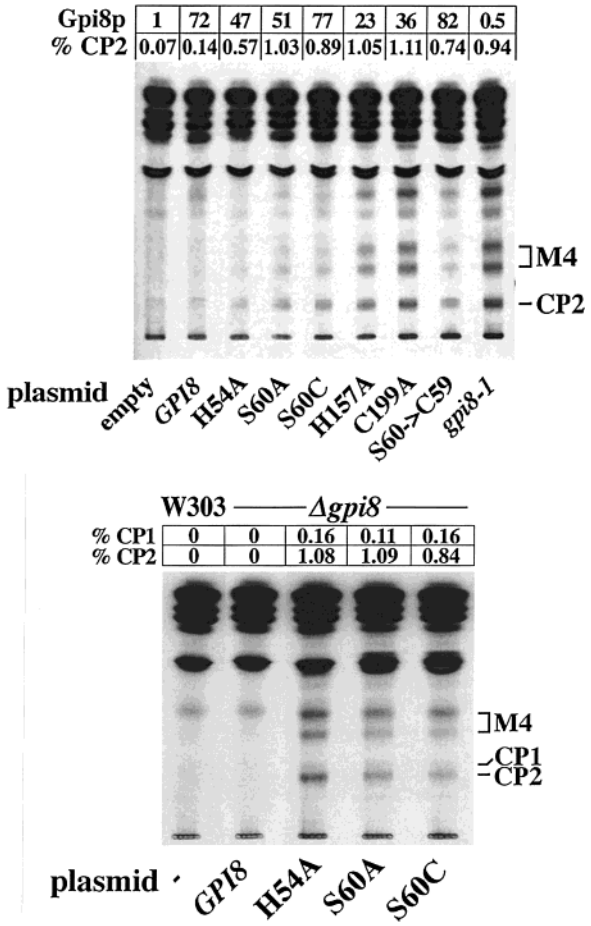

FIGURE 4: Cells harboring various mutant alleles of Gpi8p accumulate CP2. Panel a: Data shown here and in Figure 2a were obtained with cells from the very same cultures. W303 wt cells harboring pBF55-type plasmids were induced to overexpress various alleles of Gpi8p by exposure to $CuSO_4$ at 37 °C for 1 h. Thereafter, aliquots of cells were taken for metabolic labeling with [^{3}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: Δ*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}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 *GAL1-10* 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 Δ*gpi8*, the cells showed accumulation of CP2 at 37 °C, but not at 24 °C (Figure 4b and data

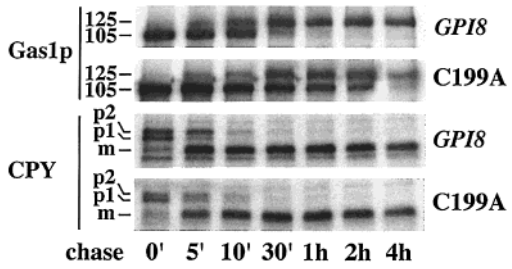

FIGURE 5: Maturation of Gas1p is delayed in wt cells overexpressing the C199A allele of *GPI8*. FBY577 and FBY578 cells harboring an integrated copy of wt *GPI8* or of C199A under the control of the *GAL1-10* promoter, respectively, were grown to exponential phase at 24 °C in selective SDaa medium. Cells 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 [^{35}S]methionine and [^{35}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 α-galactosidase and the C-terminal part of α-agglutinin (kindly provided by M. P. Schreuder; (*38*)). Among 27 000 colonies tested, none secreted detectable amounts of α-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 Δ*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 Δ*gpi8* strain containing a chromosomally integrated copy of *GPI8* under the control of the *GAL1-10* 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 *GAL1-10* 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 *GAL1-10* 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).

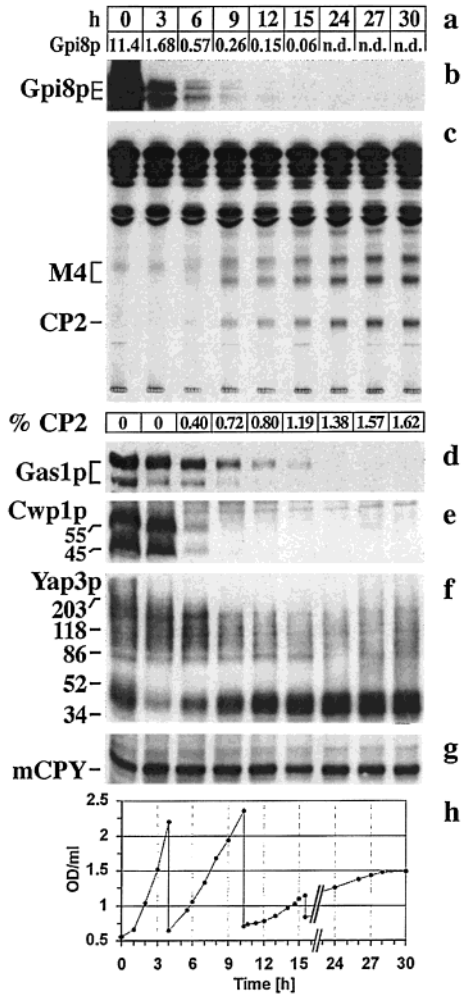

FIGURE 6: Time course of phenotypic changes during depletion of Gpi8p. FBY164 cells (Δ*gpi8/ura3-1::URA3*−*GAL1-10*−*GPI8*) were grown to exponential phase at 24 °C in SGaa medium. Expression of Gpi8p was subsequently blocked by shifting cells to glucose medium. Cultures were continued at 37 °C and periodically diluted with fresh medium to maintain the cell concentration in the range of 0.5−2.5 OD_{600}/mL. Cell growth was followed by optical density measurements (OD_{600}) and by cell counting. These two methods gave concordant results and indicated a constant ratio of $1.3-1.5 \times 10^7$ cells/OD_{600} 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 [^{3}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 [^{3}H]Ins due to a lower endogenous production of Ins at later time points, since the incorporation of [^{3}H]Ins into lipids was the same at all time points (35% of the total radioactivity

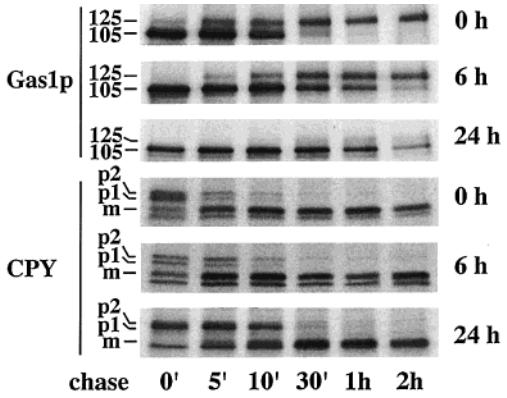

FIGURE 7: Depletion of Gpi8p blocks maturation of Gas1p. FBY164 cells were depleted of Gpi8p as described in Figure 6. Samples of the cell culture were harvested 0, 6, and 24 h after shifting to glucose. Maturation of Gas1p and CPY was analyzed at 37 °C by pulse chase labeling as described in Figure 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 from 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 34kD 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 lumenal 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 *yGPI8* 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 (ω-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 ω, $\omega+1$, and $\omega+2$ sites, but that in other positions around the ω 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 Δ*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^{N506C}, 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^{N506C} 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^{N506C}, whereas simultaneous overexpression of Gpi8p and Gaa1p

did reduce the accumulation of the immature 105 kD form of Gas1p^{N506C}, 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 Δ*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 Δ*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

We are grateful to Drs Yves Bourbonnais, Jutta Heim, Howard Riezman, Marteen P. Schreuder, and Hitoshi Shimoi for the generous gift of reagents. We are greatly indebted to Dr D. Thiele for helpful discussions and advice concerning the *CUP1* promoter expression system and to Christine Vionnet for invaluable technical assistance.

REFERENCES

1. Ferguson, M. A., and Williams, A. F. (1988) *Annu. Rev. Biochem. 57*, 285−320.
2. Conzelmann, A., Riezman, H., Desponds, C., and Bron, C. (1988) *EMBO J. 7*, 2233−2240.
3. Nuoffer, C., Jeno, P., Conzelmann, A., and Riezman, H. (1991) *Mol. Cell Biol. 11*, 27−37.
4. Wang, J., Maziarz, K., and Ratnam, M. (1999) *J. Mol. Biol. 286*, 1303−1310.
5. Conzelmann, A., Spiazzi, A., Bron, C., and Hyman, R. (1988) *Mol. Cell Biol. 8*, 674−678.
6. Conzelmann, A., Spiazzi, A., Hyman, R., and Bron, C. (1986) *EMBO J. 5*, 3291−3296.
7. Maxwell, E. S., Ramalingam, S., Gerber, L. D., Brink, L., and Udenfriend, S. (1995) *J. Biol. Chem. 270*, 19576−19582.
8. Mohney, R. P., Knez, J. J., Ravi, L., Sevlever, D., Rosenberry, T. L., Hirose, S., and Medof, M. E. (1994) *J. Biol. Chem. 269*, 6536−6542.
9. Hamburger, D., Egerton, M., and Riezman, H. (1995) *J. Cell Biol. 129*, 629−639.
10. Benghezal, M., Lipke, P. N., and Conzelmann, A. (1995) *J. Cell Biol. 130*, 1333−1344.
11. Yu, J., Nagarajan, S., Knez, J. J., Udenfriend, S., Chen, R., and Medof, M. E. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12580−12585.
12. Rawlings, N. D., and Barrett, A. J. (1993) *Biochem. J. 290*, 205−218.
13. Dalton, J. P., Hola-Jamriska, L., and Brindley, P. J. (1995) *Parasitol. 111*, 575−580.
14. Chen, J.-M., Dando, P. M., Rawlings, N. D., Brown, M. A., Young, N. E., Stevens, R. A., Hewitt, E., Watts, C., and Barret, A. J. (1997) *J. Biol. Chem. 272*, 8090−8098.
15. Kembhavi, A. A., Buttle, D. J., Knight, C. G., and Barrett, A. J. (1993) *Arch. Biochem. Biophys. 303*, 208−213.
16. Alonso, J. M., and Granell, A. (1995) *Plant Physiol. 109*, 541−547.

17. Higgins, D. G., McConnell, D. J., and Sharp, P. M. (1989) *Nature 340*, 604.
18. Eakin, A. E., Higaki, J. N., McKerrow, J. H., and Craik, C. S. (1989) *Nature 342*, 132.
19. Mottram, J. C., Coombs, G. H., and North, M. J. (1989) *Nature 342*, 132.
20. Gorbalenya, A. E., Donchenko, A. P., Blinov, V. M., and Koonin, E. V. (1989) *FEBS Lett. 243*, 103−114.
21. Higaki, J. N., Evnin, L. B., and Craik, C. S. (1989) *Biochemistry 28*, 9256−9263.
22. Rotonda, J., Nicholson, D. W., Fazil, K. M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E. P., Rasper, D. M., Ruel, R., Vaillancourt, J. P., Thornberry, N. A., and Becker, J. W. (1996) *Nat. Struct. Biol. 3*, 619−625.
23. Blow, D. M., and Steitz, T. A. (1970) *Annu. Rev. Biochem. 39*, 63−100.
24. Sprang, S. R., Fletterick, R. J., Graf, L., Rutter, W. J., and Craik, C. S. (1988) *CRC Crit. Rev. Biotechnol. 8*, 225−236.
25. Watkins, R., Weiner, L. M., and Zak, B. (1971) *Microchem. J. 16*, 14−23.
26. Harlow, E., & Lane, D. (1988) in *Antibodies: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Fankhauser, C., and Conzelmann, A. (1991) *Eur. J. Biochem. 195*, 439−448.
28. Benghezal, M., Benachour, A., Rusconi, S., Aebi, M., and Conzelmann, A. (1996) *EMBO J. 15*, 6575−6583.
29. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. te Heesen, S., Janetzky, B., Lehle, L., and Aebi, M. (1992) *EMBO J. 11*, 2071−2075.
31. Hottiger, T., Fürst, P., Pohlig, G., and Heim, J. (1994) *Yeast 10*, 283−296.
32. Becker, D. M., and Guarente, L. (1991) *Methods Enzymol. 194*, 182−187.
33. Puoti, A., and Conzelmann, A. (1992) *J. Biol. Chem. 267*, 22673−22680.
34. Sipos, G., Puoti, A., and Conzelmann, A. (1994) *EMBO J. 13*, 2789−2796.
35. Horvath, A., Sutterlin, C., Manning Krieg, U., Movva, N. R., and Riezman, H. (1994) *EMBO J. 13*, 3687−3695.
36. Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A., and Benachour, A. (1998) *Glycobiology 8*, 761−770.
37. Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A., and Benghezal, M. (1999) *J. Biol. Chem. 274*, 15251−15261.
38. Schreuder, M. P., Brekelmans, S., van den Ende, H., and Klis, F. M. (1993) *Yeast. 9*, 399−409.
39. Doering, T. L., and Schekman, R. (1996) *EMBO J. 15*, 182−191.
40. Nuoffer, C., Horvath, A., and Riezman, H. (1993) *J. Biol. Chem. 268*, 10558−10563.
41. Stevens, T., Esmon, B., and Schekman, R. (1982) *Cell 30*, 439−448.
42. van der Vaart, J. M., Caro, L. H., Chapman, J. W., Klis, F. M., and Verrips, C. T. (1995) *J. Bacteriol. 177*, 3104−3110.
43. Kapteyn, J. C., Montijn, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., Shimoi, C., Lipke, P. N., and Klis, F. M. (1996) *Glycobiology 6*, 337−345.
44. Ash, J., Dominguez, M., Bergeron, J. J. M., Thomas, D. Y., and Bourbonnais, Y. (1995) *J. Biol. Chem. 270*, 20847−20854.
45. Abe, Y., Shirane, K., Yokosawa, H., Matsushita, H., Mitta, M., Kato, I., and Ishii, S. (1993) *J. Biol. Chem. 268*, 3525−3529.
46. Chen, J. M., Rawlings, N. D., Stevens, R. A., and Barrett, A. J. (1998) *FEBS Lett. 441*, 361−365.
47. Betzel, C., Pal, G. P., and Saenger, W. (1988) *Eur. J. Biochem. 178*, 155−171.
48. Götz, B., and Klinkert, M.-Q. (1993) *Biochem. J. 290*, 801−806.
49. Gerber, L. D., Kodukula, K., and Udenfriend, S. (1992) *J. Biol. Chem. 267*, 12168−12173.
50. Micanovic, R., Gerber, L. D., Berger, J., Kodukula, K., and Udenfriend, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 157−161.
51. Moran, P., Raab, H., Kohr, W. J., and Caras, I. W. (1991) *J. Biol. Chem. 266*, 1250−1257.
52. Moran, P. and Caras, I. W. (1991) *J. Cell Biol. 115*, 1595−1600.
53. Dunn, D. E., Yu, J., Nagarajan, S., Devetten, M., Weichold, F. F., Medof, M. E., Young, N. S., and Liu, J. M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7938−7943.
54. Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T., and Takeda, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 7400−7405.
55. Gietz, R. D., and Sugino, A. (1988) *Gene 74*, 527−534.
56. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagaloff, A. (1986) *Yeast 2*, 163−168.
57. Page, R. D. M. (1996) *Comput. Appl. Biosci. 12*, 357−358.
58. Saitou, N., and Nei, M. (1987) *Mol. Biol. Evol. 4*, 406−425.

BI992186O