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Yeast Gpi8p is essential for GPI anchor attachment onto proteins

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Glycosylphosphatidylinositol (GPI) anchors are added onto newly synthesized proteins in the ER. Thereby a putative transamidase removes a C-terminal peptide and attaches the truncated protein to the free amino group of the preformed GPI. The yeast mutant *gpi8-1* is deficient in this addition of GPIs to proteins. *GPI8* encodes for an essential 47 kDa type I membrane glycoprotein residing on the luminal side of the ER membrane. *GPI8* shows significant homology to a novel family of vacuolar plant endopeptidases one of which is supposed to catalyse a transamidation step in the maturation of concanavalin A and acts as a transamidase *in vitro*. Humans have a gene which is highly homologous to *GPI8* and can functionally replace it.

Keywords: glycosylphosphatidylinositol/membrane anchors/*Saccharomyces cerevisiae*/transamidase

Introduction

Many glycoproteins of lower and higher eukaryotes are attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI) anchor (Englund, 1993; McConville and Ferguson, 1993). In man, somatic mutations in the GPI anchoring pathway of haematopoietic stem cells cause a haemolytic disease called paroxysmal nocturnal haemoglobinuria (Takeda and Kinoshita, 1995). Many protozoan pathogens such as trypanosomes, *Leishmania* or malaria parasites make extensive use of the GPI anchoring mode and the GPI pathway is presently considered as a possible target for chemotherapy. GPI anchoring is an essential process in *Saccharomyces cerevisiae* (Leidich *et al.*, 1994; Hamburger *et al.*, 1995; Leidich *et al.*, 1995; Schönbächler *et al.*, 1995; Vossen *et al.*, 1995). This organism contains ~10 functionally characterized GPI proteins many of which are located at the cell surface or are ultimately integrated into the cell wall.

The GPI anchoring pathway seems to have been elaborated early in evolution since the carbohydrate structure linking the protein to the lipid moiety is identical in all GPI anchors, namely protein-CO-NH-$(CH_2)_2$-PO_4-6Manα1-2Manα1-6Manα1-4GlcNH_2-*myo*-inositol-PO_4-lipid (McConville and Ferguson, 1993). Apart from this carbohydrate core, GPI anchors from various species differ widely in the kind of side chains attached to the core structure and in their lipid moieties.

Precursors of GPI anchored proteins have a classical signal sequence at their N terminus and a GPI anchoring signal at their C terminus; the C-terminal signal is necessary and sufficient to direct GPI addition (Caras *et al.*, 1987; Moran and Caras, 1991a; Moran *et al.*, 1991; Kodukula *et al.*, 1993). After or during import into the ER, the C-terminal GPI anchoring signal is removed and replaced by a preformed GPI. The GPI anchoring signal is composed of a C-terminal hydrophobic domain which is separated by a short hydrophilic spacer from the cleavage/attachment site (Moran and Caras, 1991b). Analysis of both, the naturally occurring attachment sites and the efficiency of GPI anchoring directed by genetically engineered sites has established a set of rules which allow prediction of the most likely GPI attachment site with >80% accuracy (Micanovic *et al.*, 1990; Moran *et al.*, 1991; Gerber *et al.*, 1992; Nuoffer *et al.*, 1993). Thus, in all species, the cleavage/attachment site (termed ω site) can only be either Asn, Ser, Gly, Ala, Asp or Cys (Micanovic *et al.*, 1990; Moran *et al.*, 1991; Nuoffer *et al.*, 1993) and the second residue at the COOH side of the ω position (ω+2) also has to be a small amino acid. Yet, the signal requirements are not identical between trypanosomes, yeast and mammalian cells so that the enzyme which presumably recognizes this signal represents a potential target for chemotherapy (Nuoffer *et al.*, 1993; Moran and Caras, 1994).

Although the transfer of preformed GPIs onto proteins can be studied by a microsomal translation–translocation–GPI anchoring system, the enzymes involved in this process are not characterized. The GPI–transferase complex is believed to act as a transamidase, i.e. to simultaneously remove the GPI anchoring signal and to transfer the preformed GPI since, 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 not only for normal cells but also for mammalian mutant cell lines unable to synthesize complete GPIs (Conzelmann *et al.*, 1986, 1988). Recently, direct evidence for a transamidase has 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 (Maxwell *et al.*, 1995). Genetic approaches also have identified genes required for addition of GPI anchors. Transamidase-deficient cells are expected to accumulate complete GPIs as well as the GPI precursor proteins. This phenotype is exhibited by two yeast mutants, *gaa1* and *gpi8* and a mammalian mutant cell line (class K) (Mohney *et al.*, 1994; Benghezal *et al.*, 1995; Hamburger *et al.*, 1995). The *GAA1* gene is essential and codes for a 68 kDa ER protein with a large luminal domain, several membrane

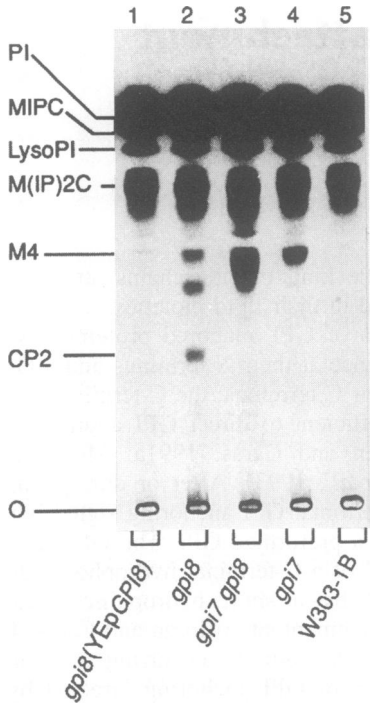

Fig. 1. Complementation of *gpi8* by plasmid YEp*GPI8*. Exponentially growing cells were radiolabelled at 37°C with [2-^{3}H]*myo*-inositol and lipid extracts were analysed by TLC and fluorography. *Gpi8-1* (lane 2) accumulates several intermediates the most polar of which is a complete GPI named CP2 [Manα1,2 (ethanolamine-PO_4→6) Manα1,2 Manα1,6 Manα1,4 GlcNα1,6 Ins1-PO_4-diacylglycerol]. *Gpi7-1* (lane 4) accumulates moderate amounts of an incomplete lipid named M4 (Manα1,2 Manα1,2 Manα1,6 Manα1,4 GlcNα1,6 Ins1-PO_4-diacylglycerol). The double mutant *gpi7-1 gpi8-1* (lane 3) accumulates very high amounts of M4. PI, phosphatidylinositol; MIPC, mannosylinositolphosphoceramide; M(IP)$_2$C, inositolphospho-MIPC.

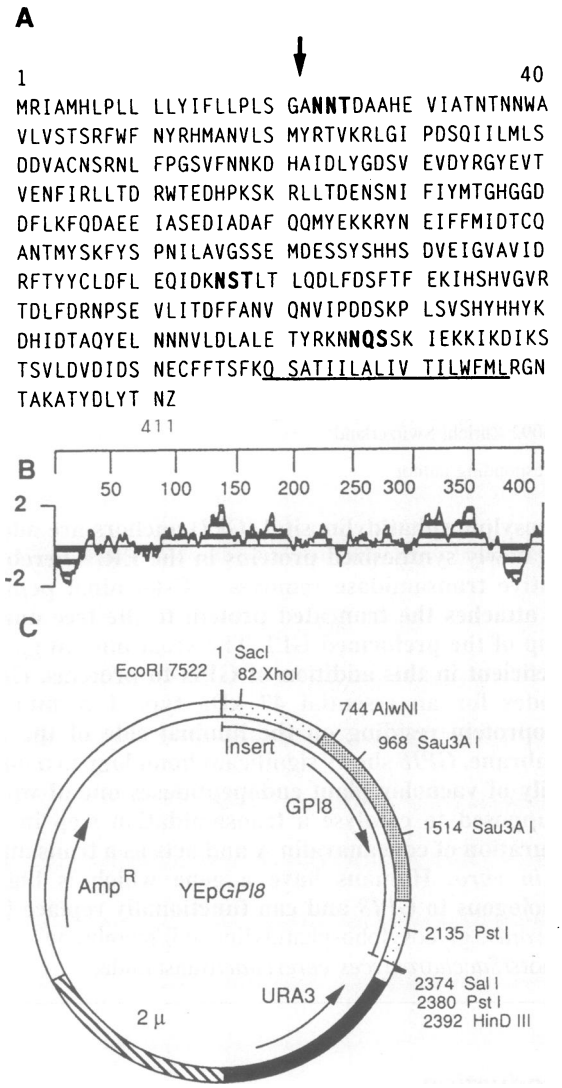

Fig. 2. Sequence of *GPI8*. (**A**) *N*-glycosylation sites are in bold face, the C-terminal transmembrane domain is underlined. The most likely cleavage site of the signal peptidase is indicated by a vertical arrow. In the hydrophilicity plot according to Kyte and Doolittle shown in (**B**), the hydrophobic sequences get a negative score. (**C**) shows the restriction map of YEp*GPI8*. The 2.4 kb insert is stippled, its open reading frame is densely stippled.

spanning domains and a cytosolic ER retrieval signal on its extreme C terminus. This protein is required for the attachment of GPIs to proteins *in vivo* although its exact role is still not elucidated.

Here we report on the cloning of *GPI8*. *GPI8* encodes a type I transmembrane ER protein with a large luminal domain. Gpi8p has 27.5% identity to a jack bean asparaginyl endopeptidase, which shows transpeptidase activity *in vitro* (Abe *et al.*, 1993). Therefore we suggest that Gpi8p could be part of the GPI–transamidase complex.

Results

Characterization of GPI8

gpi7-1 and *gpi8-1* have a defect in the synthesis and attachment of GPI anchors, respectively (Benghezal *et al.*, 1995). These two mutants still grow at 37°C, albeit more slowly. To facilitate the selection of complementing genes we crossed the two mutants and constructed a *gpi7 gpi8* double mutant. This double mutant grew well at 24°C but not at 37°C and allowed for the isolation of complementing plasmids able to suppress the accumulation of previously characterized abnormal GPI intermediates characteristic of *gpi7-1* and *gpi8-1* (Benghezal *et al.*, 1995) as shown in Figure 1. Whereas labelled lipid extracts from *gpi8-1* (lane 2), *gpi7-1 gpi8-1* (lane 3) and *gpi7-1* (lane 4) contain the characteristic intermediates, the lipid profile of *gpi8-1* harbouring a multicopy vector with a complementing insert (lane 1) is almost identical to the one of wild type cells (lane 5). Although not visible on the reproduction, the complemented *gpi8-1* strain still contains a trace of CP2 (see Discussion). The shortest complementing insert (2.4 kb) present in the multicopy plasmid YEp*GPI8* (Figure 2C) was sequenced. While sequencing was in progress, the complete sequence was submitted by the Genome Sequencing Centre at Washington University, St Louis under the gene designation D9798.2 on cosmid 9798 with GenBank accession number U32517. The sequence contains one open reading frame mapping to chromosome IV and encoding a protein of 411 amino acids of unknown function (Figure 2A). Our partial sequence covering 80% of the insert of YEp*GPI8* is completely identical with D9798.2 and YEp*GPI8* contains the same restriction sites as those predicted for D9798.2.

To create a disruption of the presumed *GPI8* gene, a DNA fragment containing the entire open reading frame

replaced by the selectable kanMX2 marker was constructed. The fragment was used to transform the diploid wild type strain FBY118 to geneticin resistance. After sporulation and tetrad dissection of one geneticin resistant transformant, only two spores of each tetrad yielded colonies whereas the two others germinated and stopped growth after 2–3 cell divisions. All viable segregants were geneticin sensitive. If the heterozygous diploid harboured the multicopy plasmid YEp*GPI8*, four colonies were obtained in each tetrad but the two geneticin resistant colonies could not lose YEp*GPI8*. This indicates that the cloned gene is essential for viability. To confirm that the isolated gene corresponds to the gene that is mutated in *gpi8-1*, the isolated gene was disrupted in the heterozygous diploid *gpi8-1/GPI8* FBY119 strain. Correct replacement of one locus was verified by whole cell PCR (Wach *et al.*, 1994) in several geneticin resistant transformants. Twenty tetrads from one of them were dissected and a 2:2 segregation of growth to non-growth was found in all tetrads. The growing segregants were geneticin sensitive and showed a wild type profile of [2-^{3}H]*myo*-inositol-labelled lipids when labelled at 37°C (data not shown). This is consistent with a deletion/insertion into the mutated *gpi8* locus of the diploid. We conclude that the cloned gene is identical to the *GPI8* locus.

GPI8 encodes a glycosylated transmembrane protein of the ER

The sequence of *GPI8* (Figure 2A) predicts 411 amino acids for a protein of 47 kDa or 45 kDa if the signal sequence is removed. The hydrophilicity plot of the Gpi8p (Figure 2B) reveals two hydrophobic regions. The region which is at the N terminus qualifies as a signal peptide for translocation into the ER (von Heijne, 1986). According to the von Heijne algorithm this domain of Gpi8p reaches a score of 10 whereas no cytosolic protein reaches a score higher than 4.4. Two cleavage sites for the signal peptidase are predicted between the residues Gly21 and Ala22 or Ala22 and Asn23. A second hydrophobic region is localized at the C terminus of the protein (Figure 2B) and is predicted to be a transmembrane helix (Rost *et al.*, 1995). We raised rabbit antibodies against a His-tagged N-terminal, soluble fragment of Gpi8p to verify these predictions. As shown in Figure 3A, this antibody reacts with four bands of 50, 48, 46 and 44 kDa. Whereas the 44 kDa band seems to be soluble, the other bands sedimented with membranes during ultracentrifugation and could be extracted from microsomes neither with 1 M sodium chloride nor by 0.1 M sodium carbonate pH 11. They were however solubilized by detergents. The size difference between the 50, 48 and 46 kDa forms of Gpi8p is due to different states of glycosylation. Upon treatment of the membrane-bound 46–50 kDa forms of Gpi8p with endoglycosidase H, Gpi8p migrates as one single band of 44 kDa (Figure 3B). This result indicates that Gpi8p contains three *N*-linked oligosaccharides and few *O*-glycans if any. The state of glycosylation of the soluble 44 kDa band (Figure 3A) has not been investigated. This band may represent a soluble degradation product of a membrane bound form or be labelled non-specifically by our antibodies.

The membrane topology of Gpi8p was investigated by a protease protection assay. If indeed the N-terminal and

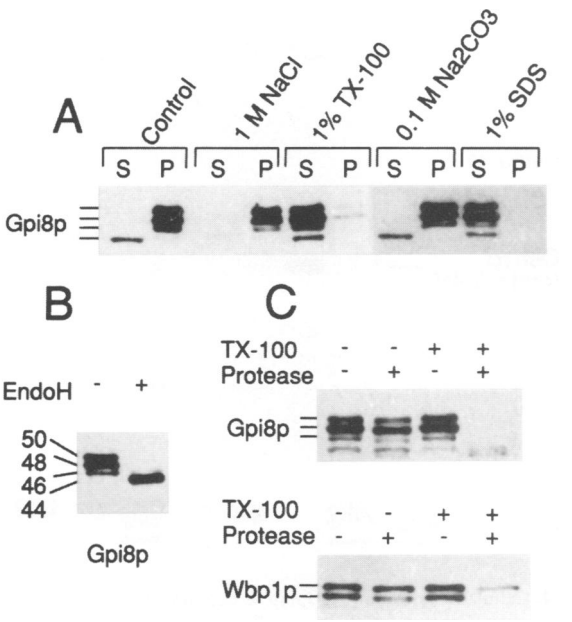

Fig. 3. Gpi8 is a luminally oriented, integral membrane glycoprotein. (**A**) Exponentially growing W303-1B were broken with glass beads and cell walls were removed. Aliquots of the lysate were incubated for 30 min at 0°C with NaCl, TX-100, Na_2CO_3 or SDS. Subsequently membranes were sedimented by ultracentrifugation. Proteins of supernatant (S) and pellets (P) were precipitated with trichloroacetic acid, processed for Western blotting and probed with affinity purified rabbit anti-Gpi8p antibodies. (**B**) A microsomal pellet from W303-1B cells was lysed in SDS and incubated with or without endoglycosidase H. The samples were processed as above. (**C**) Microsomes from exponentially growing W303-1B were digested with 0.1 mg/ml proteinase K at 0°C for 30 min in the presence or absence of 1% TX-100. Samples were processed as above and probed with rabbit anti-Gpi8p and anti-Wbp1p antibodies. Each lane in this figure contains the equivalent of two OD_{600} units of cells.

C-terminal hydrophobic domains act as a signal sequence and as a transmembrane domain, respectively, we expect that Gpi8p has a large N-terminal luminal domain and a short C-terminal cytosolic domain of ~14 amino acids. As shown in Figure 3C, neither Gpi8p nor Wbp1p could be degraded by proteinase K unless microsomes were permeabilized with Triton X-100 (TX-100). Since Wbp1p has been demonstrated to be a luminal ER protein (Sengstag *et al.*, 1990; te Heesen *et al.*, 1991), this result demonstrates that the bulk of Gpi8p is luminal. As expected, treatment of intact microsomes results in a small decrease in apparent molecular mass of Gpi8p which can be explained by the removal of the small cytosolic domain. The fact that Gpi8p is a glycoprotein confirms the proposed topology.

The cellular localisation of Gpi8p was determined by subcellular fractionation as shown in Figure 4. Gpi8p was found to co-fractionate with Wbp1p, a subunit of the oligosaccharyltransferase which resides in the ER (Sengstag *et al.*, 1990; te Heesen *et al.*, 1991). Gpi8p and Wbp1p were found exclusively in fraction P13 and upon further fractionation of P13, in fractions F3–F5, although a small amount of both of these proteins remained in F1 (see figure legend for details). Thus, Gpi8p was clearly separated from Och1p, a marker of the early Golgi which was mainly found in fraction P100 and from alkaline phosphatase (ALP) a vacuolar marker found mainly in

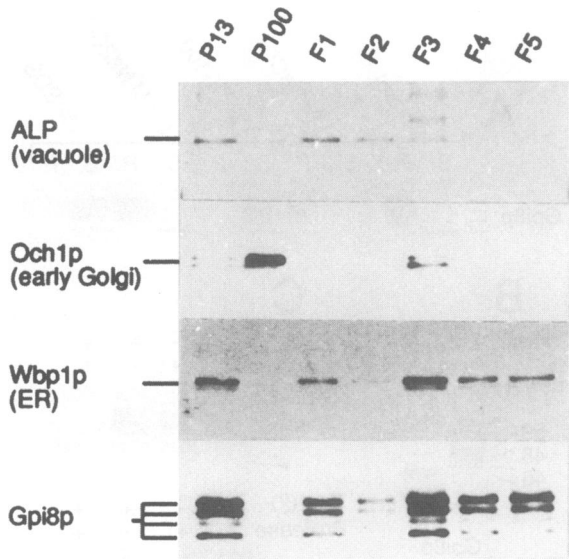

Fig. 4. Cellular localization of Gpi8p as determined by subcellular fractionation. For this experiment, a slightly modified version of a published procedure (Gaynor *et al.*, 1994) was used (see Materials and methods). In short, spheroplasts of W303-1B were osmotically lysed in a Dounce glass homogenizer, intact spheroplasts were removed by a 300 *g* spin and the lysate was subjected to differential centrifugations at 13 000 *g* and 100 000 *g*. These centrifugations generated pellet P13 containing ER and vacuolar membranes and pellet P100 which contains Golgi membranes. Pellet P13 was further resolved on a two step sucrose gradient yielding five equivalent fractions termed F1 to F5. Equivalent aliquots of all fractions were processed for SDS–PAGE and Western blotting.

fraction F1. Although fractions F3 to F5 also may contain plasma membrane Gpi8p must be localized in the ER only, since overexpression of Gpi8p results in the appearance of a hyperglycosylated form indicating that *N*-glycans of Gpi8p become elongated when Gpi8p leaves the ER (not shown). The antibody against Gpi8p was also used to demonstrate that cells harbouring vector YEp*GPI8* contain a markedly increased amount of Gpi8p and that *gpi8-1* also contains an apparently normal form of Gpi8p (not shown).

Gpi8p is homologous to a novel family of cysteine proteases.

Data base searches revealed significant homology of Gpi8p to a novel family of cysteine proteinases found in plant seeds or invertebrates. Plant proteases are Asn-specific endopeptidases and have been isolated from mature castor beans (Hara-Nishimura *et al.*, 1991, 1993), soybeans (Muramatsu and Fukasawa, 1993; Shimada *et al.*, 1994), jack beans (Abe *et al.*, 1993; Takeda *et al.*, 1994), germinating vetch (*Vicia sativa*) (Becker *et al.*, 1995), *arabidopsis thaliana* (Kinoshita *et al.*, 1995) and orange (Alonso and Granell, 1995) and are involved in the proteolytic processing of proteins in the protein-storage vacuoles. Although some of these proteases have been shown to be blocked by cysteine-alkylating reagents and therefore are classified as cysteine proteases (Abe *et al.*, 1993; Hara-Nishimura *et al.*, 1993; Shimada *et al.*, 1994; Becker *et al.*, 1995), they lack the consensus motifs of classical cysteine proteases which are in three regions centred around a Cys, a His and an Asn residue forming the catalytic triad. Gpi8p is also related to members of this novel protease family found in schistosomes (Davis *et al.*, 1987; Merckelbach *et al.*, 1994). All of these proteins have a signal sequence and exhibit strong homology except for the C-terminal parts (Figure 5A). Whereas the identity over the entire length of the proteins at the amino acid level among plant proteases amounts to ~50–75%, the identity between plant and schistosomal proteases is ~30–40% and the identity of either plant or schistosomal enzymes with Gpi8p is around 25–28%. The homology of this novel family of proteases with *GPI8* is confined to the regions which are also most strongly conserved between plant and schistosomal proteases. This suggests that the function of Gpi8p may be similar to the one of the other members of this family and supports the notion that Gpi8p could be part of the putative transamidase in yeast.

Cloning of a human homologue of GPI8

GPI8 was found to be homologous with a short cDNA sequence which has been released by the Washington University-MERCK EST (expressed sequence tag) project. This cDNA clone (GenBank accession number R18975) was obtained and was sequenced in its entire length. Its sequence predicts an open reading frame coding for a protein of 396 amino acids and of 45 kDa. The gene shows a 43% overall identity with *GPI8* and was therefore named *hGPI8* (Figure 5B). If only the N-terminal 280 amino acids of the proteins without their signal sequence are considered, the identity between *GPI8* and *hGPI8* rises to 58.5%. The homology between *GPI8* and *hGPI8* comprises most of the regions which are conserved between this novel protease family and *GPI8* (Figure 5). Moreover, *hGPI8* is as closely related to the other members of the family as *GPI8*. Also, hGpi8p has a similar hydrophilicity profile to Gpi8p with an N-terminal signal sequence and a C-terminal transmembrane domain (von Heijne, 1986; Rost *et al.*, 1995). However hGpi8 has no *N*-glycosylation sites. Indeed, when used for Western blotting on a TX-100 extract of human lymphocyte membranes, our rabbit anti-Gpi8 antibody labelled a single 47 kDa band. Thus, the apparent size of this band corresponds approximately to a molecular mass of 43 kDa which is predicted for hGpi8 from its sequence after cleavage of its signal sequence. Also an incomplete cDNA sequence showing complete sequence identity with hGpi8p over a length of 84 amino acids was isolated in a comparative EST approach to study differentiation of the rat PC-12 pheochromocytoma line and was entered into the GenBank under the accession number H34389 (Lee *et al.*, 1995).

A hybrid hGpi8p with its signal sequence replaced by the corresponding yeast signal sequence (Figure 6A) was expressed under control of the yeast promoter of *GPI8* in *gpi7 gpi8* cells. This construct was able to rescue the growth defect of the double mutant *gpi7 gpi8* at 37°C (Figure 6B). We conclude that *hGPI8* is also functional in yeast.

Discussion

We recently reported the isolation of six mutant classes involved in the GPI biosynthesis pathway (Benghezal *et al.*, 1995). A further mutant, *gpi8-1*, was found to have a ts defect in the attachment of completed GPIs to nascent

proteins. At 37°C *gpi8-1* has a reduced growth rate, accumulates the complete GPI lipid CP2 which normally remains undetectable and accumulates an immature 105 kDa ER form of the Gas1p which, when not anchored by a GPI anchor, cannot leave the ER (Nuoffer *et al.*, 1993). Also, *gpi8-1* is hypersensitive to Calcofluor White. Indeed, here we show that *GPI8* is an essential gene as are three other yeast genes necessary for an early step in the biosynthesis of GPIs, namely the addition of GlcNAc to PI (Leidich *et al.*, 1994, 1995). Whereas the growth defect and Calcofluor White hypersensitivity of *gpi8-1* were completely abolished by the presence of the multicopy vector YEp*GPI8*, the complemented mutant still accumulated a trace of CP2 which was not seen in wild type cells (Figure 1). The trace of CP2 might simply be due to cells which lost the complementing plasmid. Another possibility is that the *gpi8-1* allele somehow inhibits GPI anchor attachment, possibly through competition for some other essential subunits of the transamidase complex. The implication of this would be that in the wild type situation, these other proteins are not made in excess over Gpi8p. Alternatively, we may explain the persistence of small amounts of CP2 in the complemented *gpi8-1* by assuming that the mutation of *gpi8-1* not only abolishes the anchor attachment function but also compromises the cell's control on the GPI biosynthesis which prevents the accumulation of complete anchors in normal cells. Some support for this notion comes from a depletion experiment using a *GPI8* deletion strain maintained by a *ura3-1* locus inserted plasmid containing *GPI8* under control of the *GAL1* promoter. When this strain was shifted to glucose, it stopped growing within three doubling times but accumulated the CP2 lipid only to half the level observed in *gpi8-1* at 37°C (not shown). In view of the fact that our *gpi* mutants were identified on the basis of their ability to build up abnormal GPI intermediates, it appears conceivable that the accumulation of CP2 in *gpi8-1* is an allele-specific phenotype which is less prominent if Gpi8p is simply depleted.

Gpi8p is a luminally oriented type I integral membrane glycoprotein. Interestingly, it is not uniformly glycosylated since forms with one, two or three *N*-glycans can be detected. The protein contains three *N*-glycosylation sites, the first one being on Asn23 which is close to the most probable cleavage site for the signal peptidase (von Heijne, 1986). Cleavage between Ala22 and Asn23 would place Asn23 in a terminal position and would create an unusual site for *N*-glycosylation since both mammalian as well as yeast oligosaccharyltransferases are only minimally active on asparagines with unsubstituted α-amino groups (Bause and Hettkamp, 1979; Bause and Lehle, 1979). Thus, we propose that the signal peptidase cuts between Gly21 and Ala22 rather than Ala22 and Asn23, since the former site has almost the same probability of being the cleavage site as the latter (von Heijne, 1986) (Figure 2). We have not investigated whether the Gpi8p forms with only one *N*-glycan will mature into completely glycosylated forms

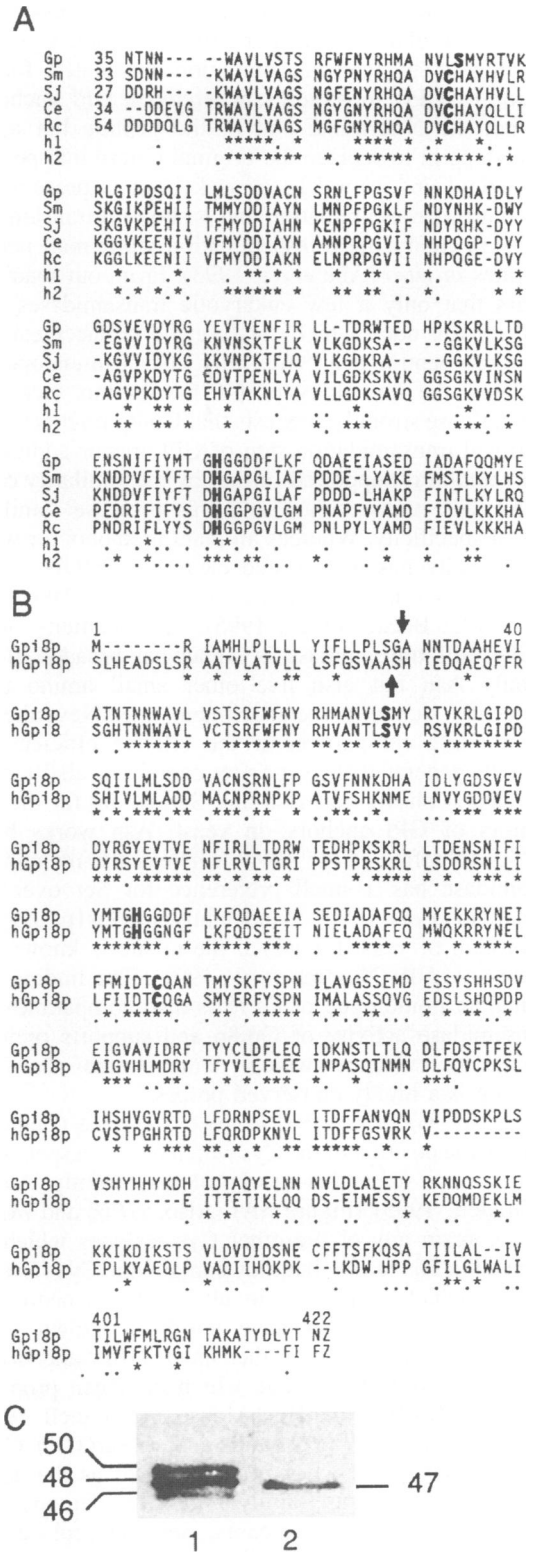

Fig. 5. Homology of Gpi8p with a novel cysteine proteinase family. (**A**) The N-terminal regions of GPI8 (Gp) and proteases from *Schistosoma mansoni* (Sm), *Schistosoma japonicum* (Sj), *Concanavalia ensiformis* (Ce) and castor beans (Rc) were aligned using the CLUSTALW multiple sequence alignments program. The sequences listed start at the positions shown at the beginning of the first block. Identities (*) and the conservative substitutions (•) among all sequences are depicted in line h1 whereas line h2 shows the same analysis when Gpi8p is excluded. Residues which might be in the active site are in bold. (**B**) shows the amino acid homology of Gpi8p and hGpi8p. The sequence of hGpi8p is incomplete at its N terminus since no start codon was found on the EST clone. (**C**) A Western blot containing an extract from W303 1B (lane 1) or 20 μg of crude membranes from human peripheral blood lymphocytes (lane 2) was probed with anti-Gpi8p antibodies.

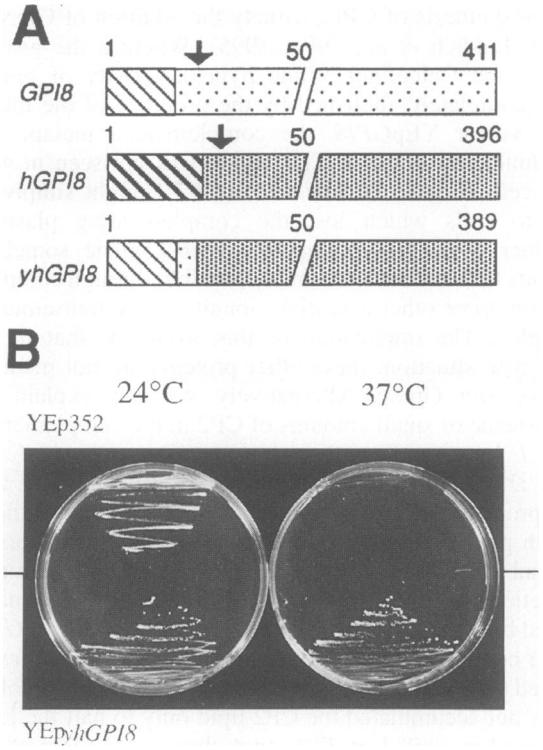

Fig. 6. *hGPI8* complements *gpi7 gpi8*. A hybrid gene containing the 5′ region of *GPI8* including its promoter plus most of *hGPI8* was constructed and cloned into YEp352 to yield YEp*yhGPI8*. The corresponding protein can be described as a hGpi8p with its signal sequence and the first six amino acids of the mature sequence replaced by the signal sequence and the first six amino acids of Gpi8p. (**A**) Signal sequences are hatched, mature sequences are stippled. Sites of cleavage for recombination are pointed out by arrows. (**B**) *Gpi7 gpi8* cells transformed with either YEp352 or YEp*yhGPI8* were streaked out on SDCA agar plates and incubated at 24 or 37°C for 48 h.

with time or if they persist in this underglycosylated state even though they reside in the same compartment as the *N*-oligosaccharyltransferase.

Subcellular fractionation shows that Gpi8p is localized in the ER. In contrast to Wbp1p and Gaa1p, which contain KKXX or KXKXX retrieval signals at their cytosolic C-terminal ends, Gpi8p does not contain any known retrieval sequence. Overexpression of Gpi8p leads to the synthesis of high molecular weight forms of Gpi8p which appear as a smear on Western blots (not shown). This material is most likely to represent heterogeneously glycosylated Gpi8p which increased in mass due to the addition of mannoses onto core oligosaccharides in the Golgi. Thus, it appears that the retention mechanism for Gpi8p is saturable. Gpi8 may be retained in the ER through an interaction with other ER resident proteins or may contain an unidentified retention motif. It is possible that Gpi8p is retained in the ER through an interaction with Gaa1p since an analysis of the codon usage in *GAA1* and *GPI8* suggests that both of these proteins are expressed at a similar level (Bennetzen and Hall, 1982). Gpi8p and Gaa1p both seem to be intimately involved in the attachment of GPI anchors to proteins since mutants in these genes produce a similar phenotype and mutations in these two genes are synthetically lethal (Benghezal *et al.*, 1995). The exact role of each of these proteins needs further examination.

It has been reported that concanavalin A, the commonly used jack bean lectin, is produced by the post-translational proteolysis and transpeptidation on the COOH side of an Asn residue close to the C terminus. After removal of the signal peptide (amino acids 1–29), the last nine amino acids at the C terminus of the precursor protein are removed and replaced by the precursor's own N terminus. Thereby the precursor is cleaved at the C-terminal side of Asn281 and the α-amino group of Ser30 is attached to the thus liberated COOH of Asn281, a process which creates a circular peptide. Formally this maturation step is analogous to the transamidation postulated for the addition of GPIs to proteins, whereby in GPI anchoring it is not the N-terminal end of the peptide but a GPI which steps in to replace the original C-terminal peptide. Asparaginyl endopeptidase of jack bean is presumed to carry out this transamidation and it shows a transamidase activity in catalyzing transpeptidation with small peptide substrates *in vitro* (Abe *et al.*, 1993). From our reading it appears that only a few eukaryotic transamidases exist and there are none which catalyse the replacement of a C-terminal peptide apart from this jack bean endopeptidase. Thus, the homology of *GPI8* with this jack bean endopeptidase strongly suggests that Gpi8p might catalyse the critical transamidation step of GPI anchor addition.

This notion is supported further by the similarity of the putative GPI transamidase and this protease family in terms of specificity. Whereas all plant members for which the specificity has been tested cleave at COOH side of Asn residues (Hara-Nishimura *et al.*, 1991, 1993; Abe *et al.*, 1993; Becker *et al.*, 1995), the specificity of the putative GPI transamidase is somewhat broader in that not only Asn but also five other small amino acids can occupy the cleavage/attachment site. Nevertheless, a quantitative comparison of the relative efficiency of artificially generated cleavage sites containing all 20 amino acids clearly shows that Asn and Ser are by far the best acceptors of GPI anchors: in yeast, Asn works better than Ser (Nuoffer *et al.*, 1993) whereas the mammalian transamidase has a small preference for Ser over Asn (Micanovic *et al.*, 1990). Incidentally, in Gas1p, the only GPI protein of yeast for which the ω site is known, the ω site is Asn506 (Nuoffer *et al.*, 1991). The finding of a mammalian homologue of *GPI8* is also compatible with a transamidase activity of Gpi8p and supports previous biochemical evidence which demonstrated that GPI anchoring is a highly conserved process.

Curiously, *GPI8* and *hGPI8* have a leucine (Leu59 of Gpi8p) in the position of the Cys which has been speculated to be the active site in this novel proteinase family (Alonso and Granell, 1995) (Figure 5A). Also, *GPI8* and *hGPI8* do not contain any of the other Cys residues which are conserved in this family but they contain one Cys (Cys199 of *GPI8*) which is present in all but the schistosomal proteases, and the latter have not been shown to be cysteine proteases. On the other hand, *GPI8* and *hGPI8* have a conserved His residue which has been proposed as the catalytically active His (Alonso and Granell, 1995). Also, Gpi8p and *hGPI8* contain a Ser (Ser60 of Gpi8) next to the Cys which has been proposed as the active site of this novel protease family. It has been demonstrated that for some cysteine proteases, Ser can replace Cys whereby some proteolytic activity is still preserved (Eakin

et al., 1989; Gorbalenya *et al.*, 1989; Higaki *et al.*, 1989; Higgins *et al.*, 1989; Mottram *et al.*, 1989). Thus, since cysteine and serine proteases contain a similar catalytic triad, it is conceivable that a protease is changing its active site from Cys to Ser during evolution. At any event, if Gpi8p is a transamidase, it is likely to have a Cys or a Ser at its active site. According to the classical enzyme mechanisms for these proteases, this active site residue would make a nucleophilic attack onto the peptide bonded carbonyl of the amino acid occupying the ω site, e.g. Asn506 of Gas1p. In this way Gas1p would become covalently bound to the active site and the resulting ester bond would have to be released by a second nucleophilic attack onto the same carbonyl by the amino group of the GPI.

Whatever the identity of the transamidase, it is likely that the enzyme-bound intermediate is shielded from the aqueous environment to prevent hydrolysis of the ester bond by water without attachment of a GPI since this kind of abortive transamidation is not observed in living cells. Also, one may speculate that the transamidase is not active unless it gets activated by the binding of a free GPI or by the interaction with another subunit which presents a GPI to the transamidase (Kodukula *et al.*, 1992). These considerations may suggest, that it will be difficult to demonstrate protease or transamidase activity of a purified enzyme. On the other hand, experiments with the microsomal *in vitro* translation–translocation–GPI addition system show that prominiPLAP, a GPI precursor protein, gets proteolytically processed at its C-terminal end without the addition of a GPI, probably through the catalytic action of the transamidase (Maxwell *et al.*, 1995). In preliminary experiments with purified Gpi8p we have so far not been able to detect any activity towards small commercial proteinase substrates such as DNP-Pro-Glu-Ala-Asn-NH_2, H-Asn-AMC and H-Asn-βNA even if millimolar concentrations of hydrazine or free ethanolamine were added as a nucleophile. Further experiments with more physiologic substrates are under way.

Materials and methods

Strains, growth conditions and materials

Saccharomyces cerevisiae strains were FBY11 (MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi8-1*), FBY15 (MATα *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi7-1*), FBY117 (MATα *ade2-1 ura3-1 leu2-3,112 his3-11,15 lys⁻*), W303-1B (MATα *ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*), W303-1A (MATa *ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*) and X2180-1A (MATa *lys⁻*). Diploid strains were FBY118 (MATa/α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 TRP1/trp1-1 his3-11,15/his3-11,15 lys⁺/lys⁻ GPI8/GPI8*) and FBY119 (MATa/α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 TRP1/trp1-1 his3-11,15/his3-11,15 lys⁺/lys⁻ GPI8/gpi8*). Cells were kept on YPD plates (Conzelmann *et al.*, 1992) and were cultured in SD medium containing salts, vitamins (but no *myo*-inositol), trace elements and 2% glucose as carbon source (Wickerham, 1946); SDC medium is the same but contains 1% casein hydrolysate in addition. SDUA, SDCUA and SDCA are SD and SDC media supplemented with uracil and/or adenine, each at 40 mg/l. The absorbance of dilute cell suspensions was measured in a 1 cm cuvette at 600 nm; 1 OD_{600} unit of cells corresponds to 1–2.5×10^7 cells depending on the strain. *Escherichia coli* strains were HB-101, XLi blue and M15. Chromogenic protease substrates were from BACHEM Feinchemikalien AG, Bubendorf, Switzerland. Antibodies to Och1p and alkaline phosphatase were kindly donated by Dr Y.Jigami (National Institute of Bioscience and Human Technology, Tsukuba, Ibaraki 305, Japan) and Dr S.Emr (Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA) respectively. A cDNA clone containing the human homologue of GPI8 was obtained from Research Genetics, Inc. (Huntsville, AL).

Generation and isolation of the gpi7 gpi8 double mutant

Strain FBY11 was crossed with FBY15 and 20 tetrads were dissected. Several tetrads contained one or two thermosensitive segregants unable to grow at 37°C. For eight tetrads containing ts segregants all the spores were radiolabelled at 37°C with [2-^{3}H]*myo*-inositol and lipid extracts were analysed by ascending TLC as described (Benghezal *et al.*, 1995). All ts clones showed a novel lipid phenotype in that they accumulated high amounts of M4 (Figure 1, lane 3). From the lipid phenotypes of spores in the eight tetrads we could establish unambiguously that all the ts clones were *gpi7-1 gpi8-1* double mutants.

Cloning and partial sequencing of GPI8

The *GPI8* gene was cloned by complementation of the temperature sensitive phenotype of the *gpi7-1 gpi8-1* double mutant. After transformation by electroporation (Becker and Guarente, 1991) with a genomic library (Stagljar *et al.*, 1994) containing partially digested yeast chromosomal DNA ligated into the vector YEp352 (Hill *et al.*, 1986), ~8000 colonies were replicated and tested for growth at 37°C. Clones able to grow at 37°C were radiolabelled at 37°C with [2-^{3}H]*myo*-inositol and lipid extracts were analysed by TLC (Benghezal *et al.*, 1995). Plasmid DNA from clones showing a lipid profile characteristic for *gpi7-1* or *gpi8-1* single mutants were isolated (Strathern and Higgins, 1991), amplified in *E.coli* strain HB-101 and tested for their ability to suppress the abnormal GPI lipid accumulation of single mutants *gpi8-1* or *gpi7-1*. A total of 17 different plasmids contained complementing activity for *gpi8-1* whereas three different plasmids complemented *gpi7-1*. All 17 plasmids complementing *gpi8-1* contained a common restriction fragment. The plasmid with the smallest complementing fragment contained a 2.4 kb insert and was termed YEp*GPI8* (Figure 2C). This insert was partially sequenced by the dideoxy sequencing method (Sanger *et al.*, 1977). The gene could be excised as a *Sac*I–*Pst*I 2.1 kb fragment (Figure 2C) which retained full complementing activity.

Disruption of GPI8 by PCR

One step disruption of *GPI8* was done as described (Wach *et al.*, 1994). Briefly, the 1.3 kb long kanMX2 module was PCR amplified by using pFA6-KanMX2 as template and adapter primers 'GPI8-forwards' (5′-tagcaaattgcaaaagccgaacaaaatgcgtatagcgatgctcgatgaattcgagctc-3′) with a 17 nucleotide homology to the pFA6-KanMX2 MCS and a 41 nucleotide homology to *GPI8* starting 25 nucleotides upstream of the start codon and 'GPI8-backwards' (5′-agtgtacaggtcataggttgctttagcagtgtttcctctcacgt-acgctgcaggtcgac-3′) with a 18 nucleotide homology to the pFA6-KanMX2 MCS and a 41 nucleotide homology to *GPI8* starting 42 nucleotides upstream of the stop codon. This PCR DNA fragment was used to transform the diploid strain FBY118, homozygous for *GPI8*, and FBY119, a heterozygous *gpi8-1/GPI8* strain. The correct targeting of the PCR-made kanMX2 module into the *GPI8* locus in geneticin resistant clones was verified by PCR with whole yeast cells using primers GPI8-minus (5′-cacatttaagcatagcaaa-3′) starting 37 nucleotides upstream of the ATG start codon, GPI8-plus (5′-cttctgtccatctatc-3′) starting 400 bp downstream of the ATG and kanMX2 (5′-gtattgatgttg-3′) (Wach *et al.*, 1994). Tetrads from both diploid strains were dissected and analysed.

Purification of recombinant His-tagged Gpi8p and antibody production

YEp*GPI8* was digested with *Sau*3AI to generate a 546 bp fragment of *GPI8* corresponding to amino acids Asp100 to Thr281 of Gpi8p. It was inserted into the bacterial expression vector pQE-31 (Quiagen) at the *Bam*HI restriction site yielding the plasmid pQE-31*GPI8*Sau. This plasmid was used to transform *E.coli*, strain *M15*. Expression of the His-tagged recombinant protein was induced with IPTG and the protein was purified on a Ni-NTA–agarose column (Quiagen) in denaturing conditions according to the manufacturer's instructions. A polyclonal antiserum was raised in a rabbit by repeated i.m. injections of 100 μg of recombinant protein. 10 mg of the recombinant protein was coupled to CNBr-activated Sepharose 4B according to instructions from Pharmacia and antiserum was affinity purified as described (Harlow and Lane, 1988).

Membrane association and glycosylation of Gpi8p

The nature of the association of Gpi8p with the membrane was investigated by following a published method (Feldheim and Schekman, 1994). To study the glycosylation of Gpi8p, W303-1B cells were grown

in YPD medium to an OD_{600} of 1–2, 100 OD_{600} units were washed once with 5 ml TEPI buffer (50 mM Tris–HCl pH 8.5, 5 mM EDTA, 2 mM PMSF, leupeptin, pepstatin and antipain, each at 30 μg/ml). 1 ml TEPI buffer and 0.3 ml of glass beads were added to the cells and cells were broken by vortexing. Supernatant was collected and centrifuged for 5 min at 300 *g* to remove unbroken cells and cell walls and the supernatant was centrifuged 1 h at 100 000 *g*. The membrane pellet was solubilized in 0.1 ml 1% SDS, heated for 5 min at 95°C, centrifuged for 10 min at 10 000 *g* and the supernatant was diluted to 1 ml with 50 mM Na-citrate buffer, pH 5. 0.5 ml aliquots of the supernatant were incubated with or without 25 mU endoglycosidase H at 37°C for 15 h. Extracts were denatured during 5 min at 95°C in reducing sample buffer and run on a 10% SDS–PAGE gel (Laemmli, 1970).

Cellular localization of Gpi8p

W303-1B were grown in YPD to an OD_{600} of 1–1.5 and were converted to spheroplasts as described (Goud *et al.*, 1988). A published procedure was adopted for subcellular fractionation (Gaynor *et al.*, 1994) with three modifications: (i) the microsomes were resuspended at 100 OD_{600} equivalents per 0.2 ml lysis buffer, (ii) we used twice the volume for the sucrose cushions and (iii) we used an AH-650 swinging bucket rotor. These modifications may be responsible for slight differences with regard to the original report: in our experiment, only minor amounts of Och1p were present in P13 and fractions F2 and F4 of the original report seem to be contained in F1 and F3 of our experiment. Protein extracts were prepared for SDS–PAGE and Western blotting using anti-Wbp1p antiserum, anti-Och1p affinity purified antiserum, anti-ALP antiserum and affinity purified anti-Gpi8p antibodies.

Complementation of gpi8-1 mutant with hGPI8

To test whether the *hGPI8* could rescue the *gpi8-1* mutant, a fusion gene was constructed. It contains the yeast *GPI8* promoter sequence plus 78 bp from the start codon of the yeast gene, corresponding to the signal sequence for ER translocation. The other part of the gene corresponds to the human gene, beginning 1083 bp upstream of the stop codon. The *Alw*NI restriction site was introduced by PCR in *hGPI8* using primers '*hGPI8*-forwardA' (5′-cagatgctgcagaacaattctttagaagt-ggcc-3′) and '*hGPI8*-reverseA' (5′-gtttggctagctgggtt-3′). The 700 bp PCR generated fragment was digested with *Alw*NI and *Afl*III. The resulting 88 bp *Alw*NI–*Afl*III fragment was cloned with the 662 bp *Xho*I–*Alw*NI fragment of YEp*GPI8* (Figure 2C) and with the 3′ part of *hGPI8* contained in a 1028 bp *Afl*III–*Nsi*I fragment from the human EST clone into YEp*GPI8* digested with *Xho*I and P*st*I. The resulting plasmid was termed YEp*yhGPI8*. This plasmid and YEp352 were introduced in the double mutant *gpi7-1 gpi8-1* and transformants were tested for growth at 24°C and 37°C.

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