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The yeast *spt14* gene is homologous to the human *PIG-A* gene and is required for GPI anchor synthesis

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The protein encoded by the yeast gene *SPT14* shows high sequence similarity to the human protein, PIG-A, whose loss of activity is at the origin of the disease paroxysmal nocturnal hemoglobinuria. The symptoms of this disease are apparently due to a loss of cell surface, glycosylphosphatidylinositol (GPI)-anchored proteins. Like *PIG-A* mutant cells, *spt14* mutant cells are defective in GPI anchoring due to a defect in the synthesis of GlcNAc-PI, the first step of GPI synthesis. The *spt14* mutant causes several other abnormalities including transcriptional defects and a downregulation of inositolphosphoceramide synthesis. We suggest that these defects are indirect results of the loss of GPI anchoring.

Key words: GPI anchor/paroxysmal nocturnal hemoglobinuria/*Saccharomyces*/*SPT14*

Introduction

Many proteins in higher and lower eukaryotes are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The structure of this anchor has been elucidated in various species and shows the common conserved core structure EtN-P-Man_3-GlcN-inositol-P-lipid. In most species the anchor cores can be modified with carbohydrate side chains and the lipid moieties can also be remodeled. Proteins to be anchored are synthesized as precursors with two cleaved hydrophobic stretches of amino acids; one at the amino-terminus which directs the protein into the secretory pathway and another at the carboxy-terminus which is an important part of the signal directing attachment of the GPI moiety. After cleavage of the precursor near the carboxy-terminus, a preformed GPI anchor precursor is added *en bloc* to the protein (Cross, 1990; Englund, 1993; McConville and Ferguson, 1993).

Processing and anchor addition are known to be sequence specific. They require a hydrophobic C-terminal domain, a small amino acid at the anchor attachment site (referred to as the ω site; Gerber *et al.*, 1992), and small amino acids in the ω + 1 and ω + 2 sites (Moran and Caras, 1991; Moran *et al.*, 1991; Nuoffer *et al.*, 1991, 1993; Gerber *et al.*, 1992; Kodukula *et al.*, 1993). Anchor biosynthesis, protein precursor cleavage and GPI anchor addition take place in the endoplasmic reticulum (Bangs *et al.*, 1985; Conzelmann *et al.*, 1987), although the exact topology of many of the reactions remains to be elucidated conclusively.

GPI anchors in *Saccharomyces cerevisiae* resemble their counterparts in other eukaryotes, but are additionally characterized by frequent remodeling of the lipid moieties to form ceramides, the presence of relatively long acyl chains (C_{26}) found in both ceramide and acyl chains of anchors, and one or two additional mannose residues on the anchor core (Conzelmann *et al.*, 1992; Costello and Orlean, 1992; Fankhauser *et al.*, 1993). In all organisms, GlcNAc-PI is first synthesized, followed by a removal of the acetyl group to form GlcN-PI. In yeast, and probably in animal cells, the GlcN-PI is then acylated on the inositol ring and the GlcN-acylPI is an obligatory intermediate in anchor biosynthesis even though the anchors found on mature GPI-anchored proteins do not contain acylated inositol (Doering *et al.*, 1989; Masterson *et al.*, 1989; Stevens and Raetz, 1991; Costello and Orlean, 1992; Stevens, 1993). In trypanosomes and toxoplasma, GlcN-acylPI is not an obligatory intermediate in GPI synthesis (McConville and Ferguson, 1993).

Little is known about the enzymes involved in synthesis of GPI or anchor attachment. In animal cells, mutants in the process of GPI anchoring have been isolated, defining several complementation groups (Hyman, 1988). The enzymatic activity encoded by one of the genes (class E mutation) has been identified and encodes dolichol-phosphomannose synthase (Chapman *et al.*, 1980). The *S.cerevisiae DPM1* gene encodes the yeast enzyme, which is also required for GPI anchoring in this organism and has been shown to complement the mammalian mutation *in vivo* (Orlean *et al.*, 1988; DeGasperi *et al.*, 1990; Orlean, 1990). A yeast mutant, defective for synthesis of GlcNAc-PI, has recently been isolated and from this study it has been suggested that GPI synthesis is essential for yeast cell viability (Leidich *et al.*, 1994). In animal cells, three complementation groups have been shown to be required for synthesis of GlcNAc-PI (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991; classes A, C and H). The class B and F mutants affect later steps of GPI biosynthesis (Puoti *et al.*, 1991; Sugiyama *et al.*, 1991).

A human somatic cell disease, paroxysmal nocturnal hemoglobinuria (PNH), has been shown to correlate with a defect in expression of GPI-anchored proteins at the cell surface of erythrocytes and probably other cells of somatic origin (Kinoshita and Takeda, 1994). Correct expression of the GPI-anchored proteins, decay accelerating factor and CD59, protect the cells from complement-mediated lysis. The gene (*PIG-A*), which when mutated causes PNH (Takeda *et al.*, 1993; Bessler *et al.*, 1994), has recently been cloned and sequenced (Miyata *et al.*, 1993). When transfected into cells from PNH patients, the gene restored

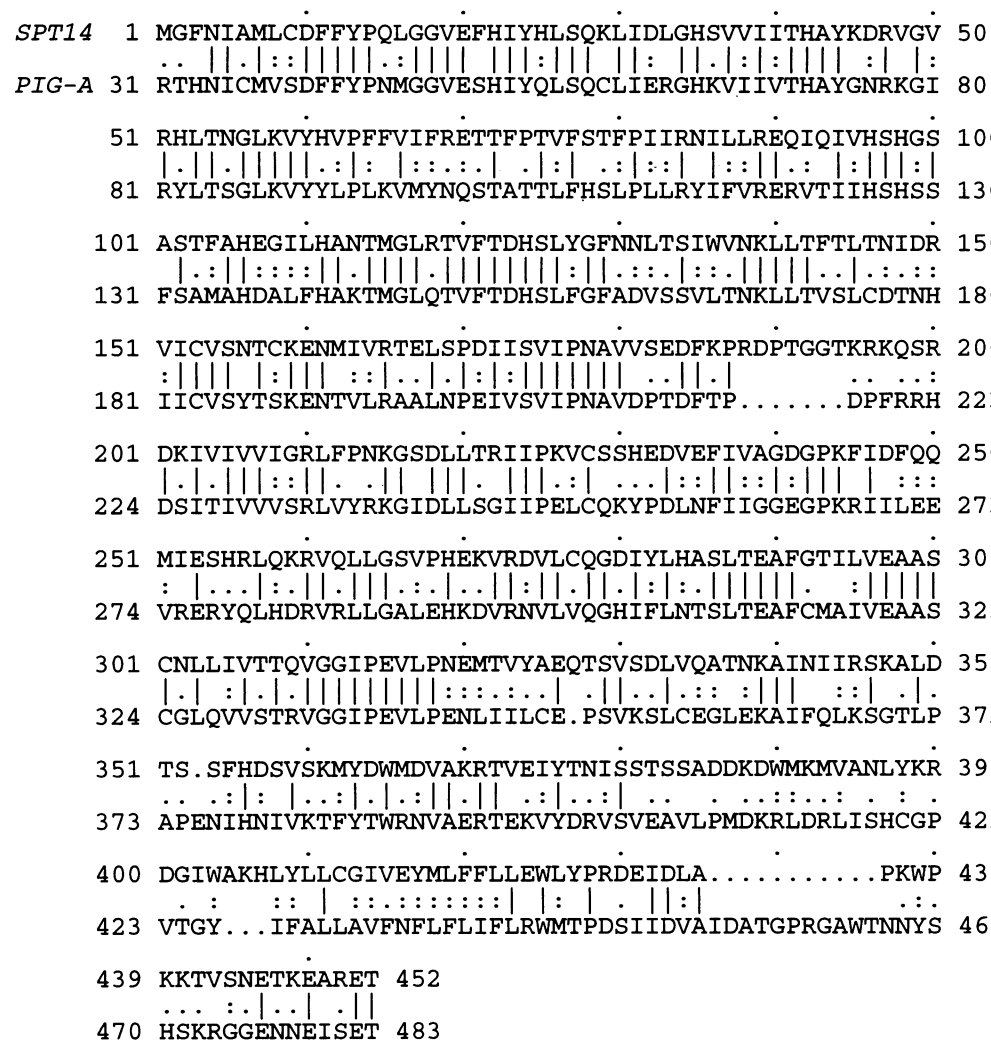

Fig. 1. Sequence similarities between Spt14p and the PIG-A protein. The Spt14 and PIG-A protein sequences were aligned using the Bestfit program of the UWGCG package. Vertical lines mark identities, dots mark similarities with double dots being higher similarity than single dots. The numbers mark the positions in the protein sequences.

synthesis of GlcNAc-PI, strongly suggesting that this gene encodes one of the proteins required to synthesize this intermediate.

In this report we study the effects of a mutation in an essential yeast gene, *SPT14*, that shows 47% identity to PIG-A at the protein level and therefore could be a homolog to *PIG-A*. Surprisingly, *SPT14* was isolated because mutations in this gene alter expression of a mutant allele of *his4* which contains a Ty element insertion in its promotor (Fassler *et al.*, 1991). This phenotype is consistent with the effects that *spt14* mutants have on transcription of the Ty transposon as well as on a number of other genes. In this report we show that the *spt14-2* mutant is severely defective in GPI anchoring, in particular in the synthesis of GlcNAc-PI. We also demonstrate a smaller, but significant, effect on ceramide synthesis in the mutant cells, which may be due to a lower expression or activity level of serine-palmitoyltransferase. We conclude that *SPT14* is a homolog of the human *PIG-A* gene and suggest that the wide-ranging pleiotropic effects seen in the mutant cells may be due to altered expression of GPI-anchored proteins and perhaps to lower levels of ceramides.

Results

Using the Bestfit program of the UWGCG package, the deduced protein sequence of the yeast gene, *SPT14*, was aligned with the human PIG-A protein sequence. The alignment is shown for the entire 452 amino acids of Spt14p (Figure 1) over which the two protein sequences show 44% identity and 66% similarity. The PIG-A protein contains an extension of about 31 amino acids at the amino-terminus. In addition to the sequence similarity, the two proteins show similar Kyte–Doolittle hydrophobicity plots (data not shown) with a putative transmembrane domain near the carboxy-terminus. The sequence of Spt14p is slightly larger at the amino-terminus than the deduced sequence originally published (Fassler *et al.*, 1991) because we have taken into account a possible splicing of the mRNA. This yields a slightly better alignment with the PIG-A protein.

spt14-2 cells are defective in GPI anchoring

When the GPI-anchored 125 kDa protein, Gas1p, does not receive a GPI anchor, it is not efficiently transported

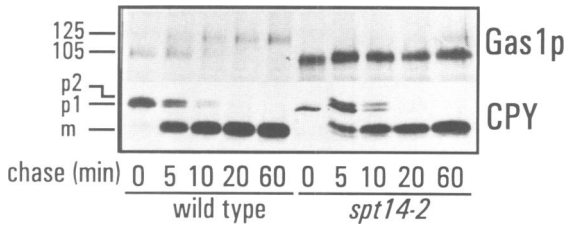

Fig. 2. Biogenesis of Gas1p and CPY in wild-type and *spt14* mutant cells. Wild-type and mutant cells were pulse labeled for 5 min with [^{35}S]methionine and cysteine, then chased with the unlabeled amino acids. Gas1p and CPY were then immunoprecipitated from cell lysates and separated on 7.5 or 10% SDS–polyacrylamide gels, respectively, dried, prepared for fluorography and exposed to X-ray film at −70°C. The time of chase is indicated, as well as the position of migration of the various forms of the proteins described in the text.

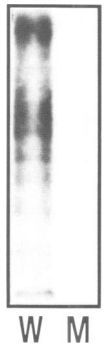

Fig. 3. [^{3}H]Inositol labeling of proteins in wild-type and *spt14* mutant cells. Wild-type and mutant cells were labeled with [^{3}H]inositol as described in Materials and methods and total proteins were extracted and separated by SDS–PAGE on a 7.5% gel. The gel was prepared for fluorography and exposed at −70°C. W, wild-type cells; M, *spt14-2* mutant cells.

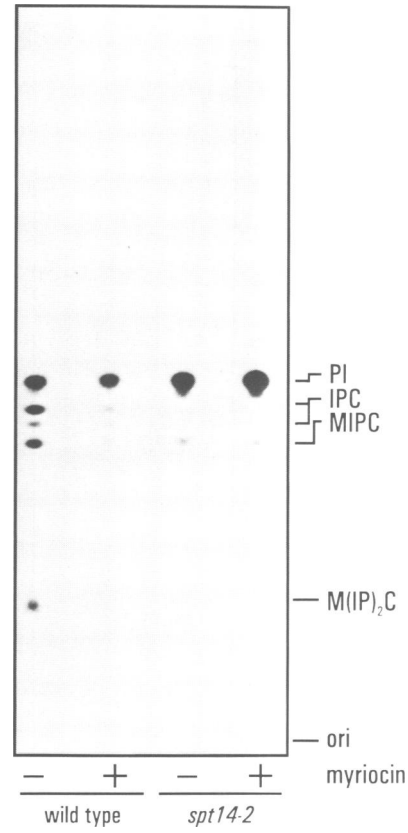

Fig. 4. [^{3}H]inositol labeling of lipids in wild-type and *spt14* mutant cells. Wild-type and *spt14-2* mutant cells were labeled with [^{3}H]inositol in the presence (+) or absence (−) of myriocin as described in Materials and methods. The lipids were extracted and separated by TLC, and the TLC plate was dried, sprayed with enhancer and exposed for fluorography. The positions of migration of phosphatidylinositol (PI), inositolphosphoceramides (IPC), mannosylinositolphosphoceramide (MIPC), mannosyldiinositol-phosphoceramide (M(IP)$_2$C) and the origin (ori) are shown.

to the Golgi apparatus and remains in its immature 105 kDa form (Nuoffer *et al.*, 1993). Therefore, to obtain an initial indication as to whether *spt14* cells are defective in GPI anchoring, we examined the maturation of Gas1p in the *spt14-2* mutant and wild-type cells by pulse–chase labeling with [^{35}S]methionine and cysteine, followed by immunoprecipitation and analysis by SDS–PAGE (Figure 2). After pulse labeling, Gas1p appeared in both mutant and wild-type cells in its 105 kDa ER form which represents the core glycosylated protein. After 5 min of chase in wild-type cells, maturation of Gas1p to the 125 kDa form began, and was completed by 20 min of chase. In *spt14-2* cells, maturation of Gas1p was largely defective with only a small amount of maturation seen after 60 min of chase. The strong delay in maturation of Gas1p could be due to a defect in GPI anchoring of the protein, entailing a block in transport to the Golgi, or simply to a general defect in protein transport. In order to test whether transport to the Golgi is generally affected in *spt14-2* cells, the same extracts were precipitated with antibodies against carboxypeptidase Y (CPY; Figure 2). After pulse labeling, most of the CPY was in its 67 kDa ER form (p1), which was chased into the 69 kDa Golgi form (p2) and finally matured in the vacuole by proteolytic cleavage to the 61 kDa mature form (m) (Stevens *et al.*, 1982). The kinetics of maturation of CPY were virtually identical in wild-type and *spt14-2* cells, even though the latter have a much longer generation time. These results suggest that the reason for the delay in maturation of Gas1p in *spt14-2* cells is a lack of GPI anchoring.

In order to prove that GPI anchoring is in fact defective in *spt14-2* cells and to show that the effect is not restricted to Gas1p, we labeled wild-type and mutant cells with [^{3}H]myoinositol. All detectable radioactive inositol incorporation into proteins in *S.cerevisiae* is due to GPI anchoring (Conzelmann *et al.*, 1990). Therefore, if the GPI anchoring defect is general, then virtually no [^{3}H]inositol should be incorporated into proteins in *spt14-2* cells. This was indeed the case (Figure 3).

As a control that *spt14-2* cells are capable of utilizing [^{3}H]inositol, we examined its incorporation into total lipids in wild-type and mutant cells. The total incorporation of [^{3}H]inositol into lipids in wild-type and *spt14-2* cells was approximately the same, but surprisingly the pattern of incorporation was not identical (Figure 4). Incorporation into phosphatidylinositol (PI) was slightly greater in the *spt14-2* cells, but incorporation into the inositolphosphoceramides (IPCs) was significantly reduced. Two lines of evidence suggest that the spots which are reduced in the mutant cells corresponded to IPCs. First, incorporation of inositol into these spots was reduced by addition of myriocin (Figure 4), an inhibitor of serine-palmitoyltransferase (see below). Second, the spots which were

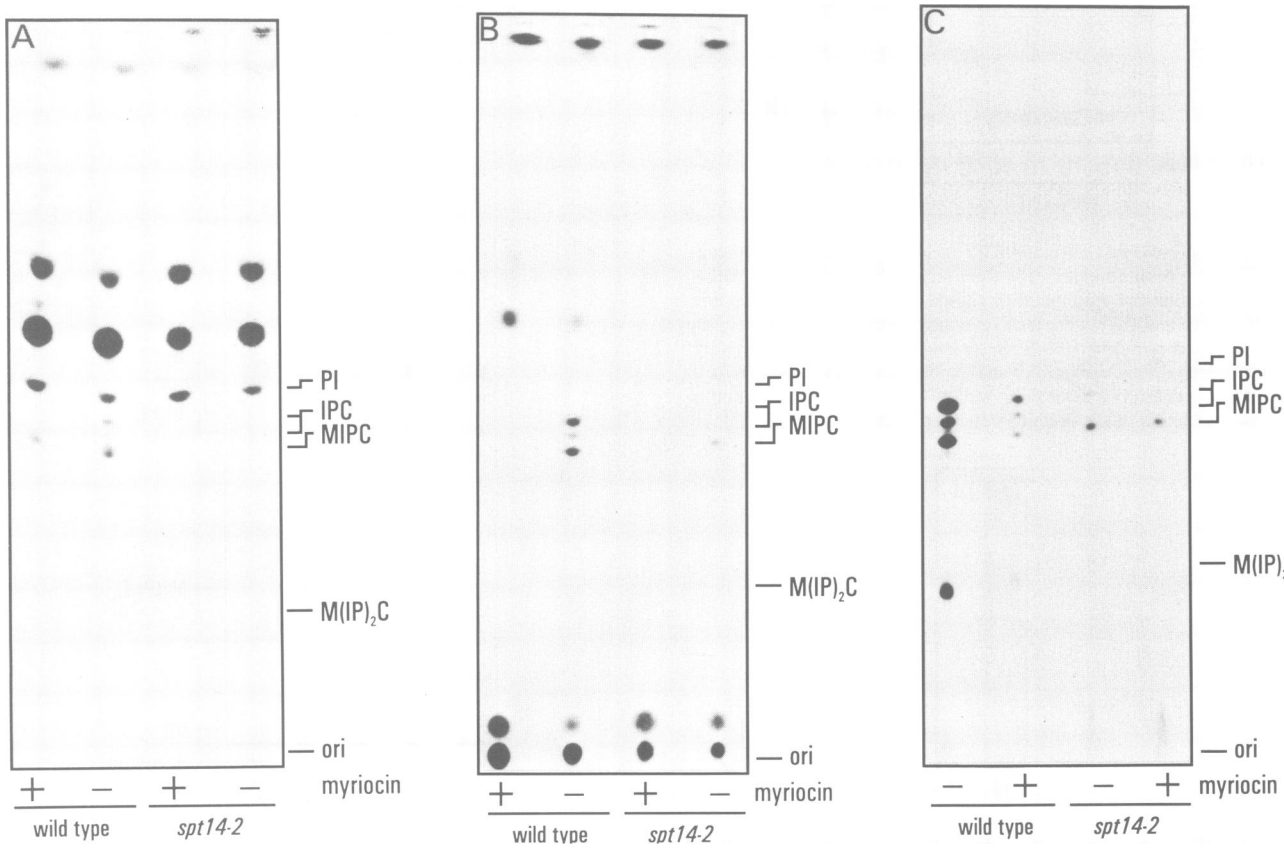

Fig. 5. Serine and inositol labeling of wild-type and mutant cells. Wild-type and mutant cells were labeled in the presence (+) or absence (−) of myriocin with [^{3}H]serine (**A** and **B**) or [^{3}H]inositol (**C**) and lipids were extracted. The lipids were separated by TLC before (A) or after (B and C) treatment with mild base. The positions of migration of inositol-labeled standards are marked as in Figure 4. The spots appearing just above the migration point of PI found in extracts from wild-type cells (stronger with myriocin) are the results of incomplete cleavage by the base treatment. In other experiments, they were completely digested.

reduced in intensity in the *spt14-2* mutant cells were also resistant to hydrolysis by mild base treatment (Figure 5C), a hallmark of the yeast IPCs. These results confirm that GPI anchoring is severely defective in *spt14-2* cells and suggest that the synthesis of IPCs is downregulated in the mutant.

As both IPC synthesis and GPI anchoring utilize PI as a substrate, we examined whether the *spt14-2* mutation could be affecting an enzymatic activity shared between the two pathways. The major step utilizing PI in IPC synthesis is the putative transfer of inositol-P from PI onto ceramide (Lester and Dickson, 1993). If this step were specifically inhibited, then one would expect to find an accumulation of ceramide and/or precursors to ceramide in the mutant cells. In order to look for this we labeled cells with [^{3}H]serine. Serine is transferred in the first step in sphingolipid biosynthesis to palmitate (from palmitoyl-CoA) to form 3-ketodihydrosphingosine (KDS). Thus, besides labeling several phospholipids, [^{3}H]serine labels all sphingolipids and their precursors. Labeling with serine confirmed the results with inositol; serine incorporation into base-sensitive phospholipids was normal in *spt14-2* cells, but the mutant cells made less IPCs than wild-type cells (Figure 5). In *spt14-2* cells an extra serine-labeled spot was detected near the front. This spot was not base-resistant. No base-resistant IPC precursors could be found. These results suggest that the reason for the lower level of IPC synthesis in *spt14-2* cells is not likely to be due to a common use of PI in the two pathways.

spt14-2 cells show lowered serine-palmitoyl-transferase activity

The finding that no ceramide precursors were found to accumulate in the *spt14-2* mutant suggested that the activity of the first enzyme in the pathway, serine-palmitoyltransferase, could be affected in mutant cells. In order to test this we assayed for this enzymatic activity in crude membrane preparations from wild-type and *spt14-2* mutant cells. In the assay buffer we included [^{3}H]serine and palmitoyl-CoA as substrates and pyridoxal−phosphate as a cofactor (see Materials and methods). When membranes were incubated with labeled serine for 30 min at 30°C, a labeled, palmitoyl-CoA-dependent spot was evident on TLC plates (Figure 6). This spot was presumably KDS because its appearance depended upon addition of palmitoyl-CoA and serine, no NADPH was added which is required for the subsequent reaction, and because the appearance of the spot was inhibited by adding myriocin during the assay. Quantification performed on separate experiments showed that the appearance of this spot was inhibited by greater than 98% by myriocin, a structural analog of sphingofungins, known inhibitors of serine-palmitoyltransferase (Zweerink *et al.*, 1992). Membranes from *spt14-2* cells had only 20% of the serine-

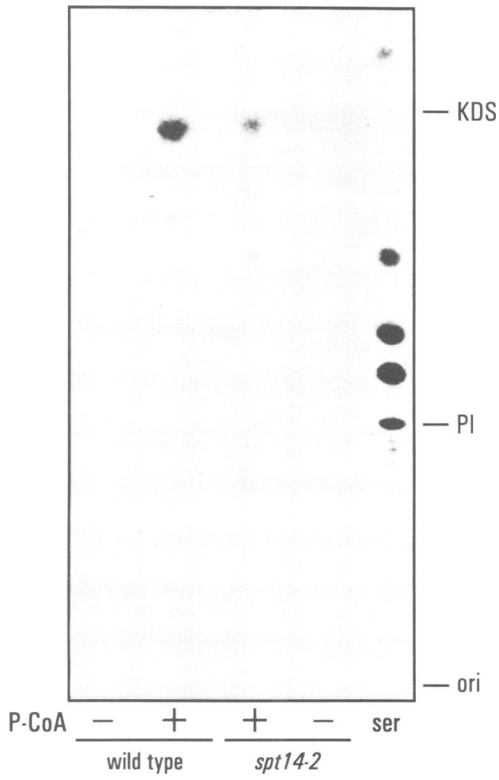

Fig. 6. *In vitro* assay of serine-palmitoyltransferase. Membranes were prepared from wild-type and *spt14-2* mutant cells and incubated under appropriate conditions to measure serine:palmitoyltransferase (Materials and methods). The end products were extracted and separated by TLC, the plate sprayed with enhancer and exposed to film at −70°C. A specificity control for this assay was the omission of one of the substrates, palmitoyl-CoA. [^{3}H]serine lipids from an *in vivo* labeling were run for comparison (ser). Scanning densitometry of a film from another experiment showed that incubation of wild-type membranes with the serine-palmitoyltransferase inhibitor, myriocin, inhibited the appearance of ketodihydrosphingosine (KDS) by >98%.

palmitoyltransferase specific activity as membranes from wild-type cells as determined by densitometric scanning of the fluorogram, confirming the reduction in this activity in the mutant cells.

GlcNAc-PI synthesis is defective in spt14-2 membranes

In order to test whether *spt14-2* membranes are defective in the same enzymatic step as class A GPI anchoring mutants in animal cells we assayed production of GlcNAc-PI, GlcN-PI and GlcN-acylPI by isolated membranes from wild-type and mutant cells. To assay these steps, we used the same crude membrane preparation as above, incubated with [^{3}H]UDP-GlcNAc at 30°C for 30 min. To inhibit production of dolichol-P-P-GlcNAc (Lehrman, 1991) and therefore *N*-glycan synthesis, we added tunicamycin to the assay buffer in certain reactions.

When tunicamycin was included in the reaction mixture, *spt14-2* membranes showed extremely low or no incorporation of [^{3}H]UDP-GlcNAc into spots comigrating with GlcNAc-PI and GlcN-PI in comparison with wild-type membranes (Figure 7). Addition of palmitoyl-CoA and ATP allowed the synthesis of GlcN-acylPI by wild-type, but not mutant membranes. However, when tunicamycin was omitted, two spots that comigrated

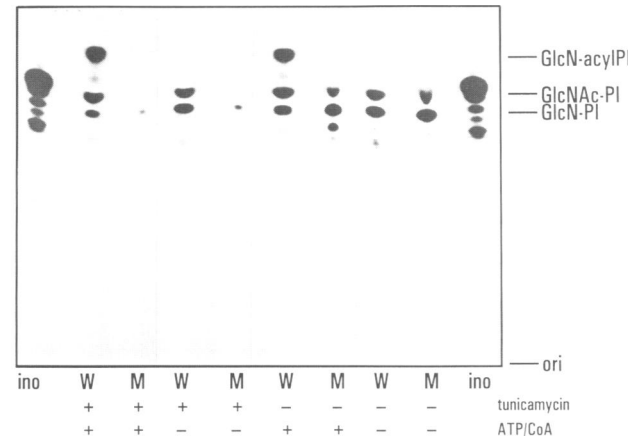

Fig. 7. *In vitro* assay of the initial steps of GPI synthesis. Membranes were prepared from wild-type (W) or *spt14-2* mutant (M) cells and incubated in the presence (+) or absence (−) of tunicamycin and/or ATP plus CoA and radiolabeled UDP-GlcNAc. The glycolipid products were extracted and separated by TLC, and the plate was sprayed with enhancer and exposed to film at −70°C. The presumptive position of migration of GlcN-acylPI, and the position of migration of standards, GlcNAc-PI, and GlcN-PI are marked.

approximately with GlcNAc-PI and GlcN-PI were detected in the mutant membranes, suggesting that these spots were dolichol-P-P-GlcNAc or derived from it, and that the initial steps of the core *N*-glycan synthesis are normal in the mutant. Indeed, all of the products synthesized by mutant membranes from UDP-GlcNAc in the presence of tunicamycin were sensitive to cleavage by mild acid treatment (data not shown), consistent with them being dolichol derivatives. GlcNAc-PI and GlcN-PI are not sensitive to cleavage by mild acid. Without tunicamycin, synthesis of GlcN-acylPI by wild-type membranes was detected because this compound migrates faster than the putative dolichols. GlcN-acylPI was not synthesized by mutant membranes in the absence of tunicamycin. The lack of effect of the *spt14-2* mutation on dolichol-P-P-GlcNAc synthesis is consistent with the lack of effect of the mutation on N-linked glycosylation (see Figure 2) and demonstrates the specificity of the defect found *in vitro* for GlcNAc-PI synthesis. The identification of GlcN-PI and GlcNAc-PI was confirmed by comigration with standards, by their sensitivities to PI−PLC cleavage (Figure 8A) and by the sensitivity of the former, but not the latter, to nitrous acid cleavage (Figure 8B). The identification of GlcN-acylPI was confirmed by its appearance only when ATP and CoA were added, by its insensitivity to PI−PLC cleavage (Figure 8A) and by its sensitivity to nitrous acid cleavage (Figure 8B). Therefore, we conclude that *spt14-2* cells and membranes show a severe defect in GlcNAc-PI synthesis, the same enzymatic step at which the PIG-A protein is implicated.

The possibility still exists that the *spt14-2* mutant is defective in GlcNAc-PI synthesis due to the defect in IPC biosynthesis. This could be the case if the pathway of GlcNAc-PI synthesis in yeast were PI→IPC→GlcNAc-IPC→GlcNAc-PI. If this pathway is correct, then [^{3}H]inositolPC would be the precursor to GlcNAc-P[^{3}H]inositol, and the synthesis of the latter compound should be strongly blocked by myriocin through its inhibition of IPC synthesis. To test this we labeled the yeast *gaa1*

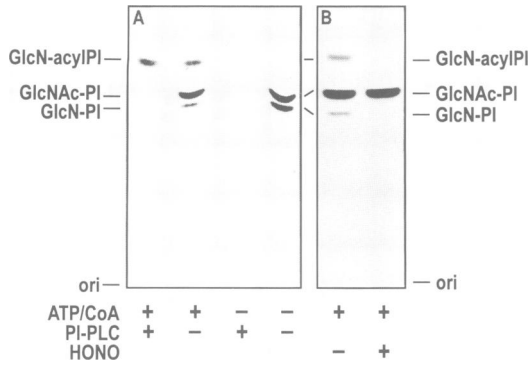

Fig. 8. Enzymatic and chemical treatments. Membranes from wild-type cells were incubated in the presence of tunicamycin, with or without ATP plus CoA and radiolabeled UDP-GlcNAc as in Figure 7. The glycolipid products were extracted and (**A**) treated with or without PI−PLC or (**B**) treated with or without nitrous acid, then reextracted, separated by TLC, and displayed as in Figure 7.

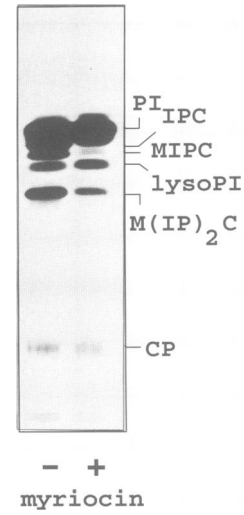

Fig. 9. GPI synthesis is not inhibited by myriocin. Strain RH401–7C was grown overnight at 24°C and labeled at 37°C with [^{3}H]inositol for 40 min in the presence or absence of myriocin. Lipids were extracted, separated using chloroform−methanol−water (10:10:3) by TLC and displayed as in Figure 7. CP marks the position of migration of the complete GPI precursor, CP2.

mutant with [^{3}H]inositol in the presence and absence of myriocin, extracted and separated the labeled lipids by TLC. This mutant synthesizes the complete GPI precursor (CP) as previously identified (Sipos *et al.*, 1994), but does not attach it to proteins (Hamburger *et al.*, 1995). This allows facile detection of the incorporation of radiolabeled inositol into CP which is normally difficult to detect in yeast (Sipos *et al.*, 1994). As can be seen in the autoradiogram shown in Figure 9, myriocin almost completely inhibited the synthesis of IPC and MIPC, strongly inhibited the synthesis of $M(IP)_2C$, but only slightly affected inositol incorporation into CP. Densitometric scanning of different autoradiograms derived from this TLC plate showed that without myriocin, ~0.14% of the total [^{3}H]inositol-labeled lipids synthesized in the *gaa*1 mutant were CP. In the presence of myriocin this amount was ~0.12%. These data show that IPC is not required as a precursor for GlcNAc-PI and strongly suggest that the lack of GlcNAc-PI synthesis in the *spt14-2* mutant is not due to the defect in ceramide synthesis. Therefore, we conclude that *SPT14* is a true homolog of PIG-A because it is required at the same step in yeast and man, that is the synthesis of GlcNAc-PI.

Discussion

Here, we present evidence that the yeast gene, *SPT14*, is a homolog of the human gene, *PIG-A*, which is at the origin of the somatic cell disease, paroxysmal nocturnal hemoglobinuria (PNH). The major line of evidence suggesting this homology is that the yeast *spt14-2* mutant is defective in GPI anchoring, and in particular, in the formation of GlcNAc-PI, like cells from patients with PNH (Takahashi *et al.*, 1993). The proteins encoded by the two genes are highly homologous, with 44% identity and 66% similarity. This homology is spread over the entire sequence of Spt14p, suggesting not only conservation of a particular functional domain, but that the proteins are true homologs.

However, a GPI anchoring defect is not the only defect found in *spt14-2* cells. They have a very long doubling time, transcriptional abnormalities (Fassler *et al.*, 1991), cell separation defects (data not shown) and a lower rate of sphingolipid synthesis than wild-type cells. The *SPT14* gene is essential for growth in yeast (Fassler *et al.*, 1991), in contrast to *PIG-A* or GPI anchoring mutants in animal cells (Hyman, 1988; Takahashi *et al.*, 1993), where GPI anchoring does not seem to be essential, in some cell types. The *SPT14* gene is likely to be an essential gene owing to its role in GPI anchoring because another gene, *GPI1*, is also essential and required for GPI anchoring (Leidich *et al.*, 1994). It is not yet clear whether the *SPT14* gene has multiple functions or whether all of the demonstrated phenotypes are a result of the block in GPI anchoring, but we favor the latter explanation.

The severe nature of the *spt14-2* mutation could alter the expression of several GPI-anchored proteins. In addition, recent evidence from our laboratory suggests a specific cotransport of GPI-anchored proteins and sphingolipids to the Golgi in yeast (Horvath *et al.*, 1994). The cotransport of GPI-anchored Gas1p depended upon the presence of the glycolipid anchor on the protein, which could be explained if sphingolipids physically interacted with GPI anchors during transport. When GPI-anchored proteins are not efficiently anchored, they are not transported to the Golgi (Micanovic *et al.*, 1990; Moran and Caras, 1992; Nuoffer *et al.*, 1993). Thus, it is possible that in the absence of transport of GPI-anchored proteins to the Golgi and subsequently to the cell surface, that a partial defect in transport of sphingolipids to these locations might also ensue. This could result in two separate effects. First, the absence or depletion of GPI-anchored proteins from the cell surface and altered surface sphingolipid content could alter the properties of this membrane. The lipid and sphingolipid composition of the plasma membrane (Patton and Lester, 1991) has been shown to be very important for many functions (Gaber *et al.*, 1989; Patton and Lester, 1992), and therefore its alteration could be the reason for the many of the pleiotropic effects seen in *spt14-2* cells, even altered gene expression.

Second, under normal conditions newly synthesized IPCs are rapidly depleted from the endoplasmic reticulum

(Horvath *et al.*, 1994). If, in the mutant, they remain for an extended period of time in this organelle, they could regulate their own synthesis by feedback regulation of the expression of serine-palmitoyltransferase, or by feedback inhibition of the enzyme. Some aspects of this explanation for the pleiotropic effects of *spt14-2* can be tested, for instance the regulation of serine-palmitoyltransferase by IPCs. It is interesting to note that the other known thermosensitive GPI anchoring mutant, *gpi1*, also shows reduced incorporation of inositol into MIPC at non-permissive temperature, supporting this hypothesis (Leidich *et al.*, 1994). As stated in the Results, it is highly unlikely that the defect in GPI synthesis in *spt14-2* cells is due to the defect in ceramide biosynthesis, because inhibition of the ceramide synthesis using myriocin had very little effect on GPI synthesis. Even though we consider it unlikely, we cannot formally rule out that Spt14p acts more directly to control expression of serine-palmitoyltransferase and enzymes required for GlcNAc-PI synthesis.

The *spt14-2* mutant membranes are defective in the synthesis of GlcNAc-PI. Other complementation groups of yeast mutants have been isolated that affect the same step in GPI synthesis (Leidich *et al.*, 1994). In mammalian cells, three complementation classes have been found that are defective in this reaction (Stevens and Raetz, 1991; Sugiyama *et al.*, 1991). This raises the question of the precise function of these three gene products. Perhaps the reaction is a simple, single enzymatic step and a three-subunit enzyme is required to perform it. This would be rather different from the known glycosyltransferases where single proteins are responsible. Alternatively, the reactions that synthesize GlcNAc-PI (*N*-acetylglucosaminyltransferase), GlcN-PI (deacetylase) and GlcN-acylPI (acylase) could be part of a single enzyme complex and mutations in any of the enzymatic activities would affect the activity of the others if the reactions are coupled together in some way or the assembly of the enzyme is affected when one of the subunits is defective. An enzymatic complex with multiple activities could be an efficient way to concentrate and funnel substrates that cannot, to our current knowledge, be used in other pathways. The precise assignment of enzymatic function to the individual polypeptides required for synthesis of these initial products of GPI synthesis may require purification of the enzymatic machinery.

Materials and methods

Cell cultures and media

The yeast mutant strain RH329–3B (*MATa spt14-2 ura3 his4 leu2*) and corresponding wild-type RH329–3C (*MATa trp1::URA3 ura3 his4 leu2*) were routinely grown in YPUD [1% yeast extract (Gibco), 2% peptone (Gibco), 30 mg/l uracil, 2% glucose], overnight at 24°C and harvested in log phase ($<2\times10^7$ cells/ml). For most biosynthetic labeling experiments, cells were labeled in SD medium with or without inositol (Dulić *et al.*, 1991), and pregrown overnight in SD medium containing 0.2% yeast extract (SDYE). For the labeling of strain RH401-7C (*MATα gaa1-1 ura3 leu2*), the cells were pregrown in SDCU medium (Sipos *et al.*, 1994).

Pulse–chase experiments

Pulse–chase labeling experiments (Horvath *et al.*, 1994) were carried out with cells grown overnight on SDYE at 24°C, harvested in aliquots of 2.5×10^7 cells per sample, and washed twice with SD medium. The cells were resuspended in 1 ml of SD medium and preincubated at 30°C for 10 min. A 5 min pulse was initiated by adding 100 μCi of a mixture of radiolabeled methionine and cysteine (EXPRE^{35}S^{35}S, NEN-Dupont). The pulse labeling was terminated by adding 10 μl of 100× freshly made chase solution [0.3% methionine, 0.3 M $(NH_4)_2SO_4$, 0.3% cysteine]. The reaction was stopped at the desired time points by adding NaF and NaN_3 to a final concentration of 10 mM each and placing the cells on ice. After termination of the pulse–chase labeling, the cells were collected by centrifugation, resuspended in 250 μl TEPI (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 2 mM phenylmethylsulfonylfluoride and 30 μg/ml each of chymostatin, leupeptin, antipain and pepstatin diluted freshly from a 100× stock in DMSO), and were then broken by vortexing for a total of 4 min for 1 min intervals with glass beads (0.45 mm diameter). Care was taken to keep the cells on ice for at least 1 min between vortexing intervals. The glass beads were allowed to settle and the supernatant was saved. The beads were washed with another 250 μl of TEPI and to the pooled supernatants, 50 μl of 10% SDS was added, the solution was mixed by vortex and immediately heated for 5 min at 95°C. Insoluble components were removed by centrifugation and the remaining supernatant was diluted with 5 ml of TNET (30 mM Tris–HCl pH 7.5, 120 mM NaCl, 5 mM EDTA, 1% v/v Triton X-100). The lysate was then incubated either overnight at 4°C or for 2 h at room temperature with rabbit antisera raised against Gas1p (Nuoffer *et al.*, 1991) or carboxypeptidase Y (CPY) (Raths *et al.*, 1993). The immune complexes were then precipitated with protein-A sepharose beads, which were sedimented at low speed and washed several times with TNET. For analysis of immunoprecipitates, the beads were resuspended in SDS–sample buffer, heated to 95°C for 5 min and after centrifugation, the supernatant was analyzed by SDS–PAGE (Laemmli, 1970) .

Labeling with [^{3}H]myoinositol and [^{3}H]serine

Cells were grown overnight on SDYE at 24°C. From that culture 1.5 OD_{600} units were harvested and washed twice with SD. The cells were then resuspended in SD supplemented with appropriate amino acids and vitamins, but lacking inositol and incubated 10 min (protein labeling) or 20 min (lipid labeling) at 30°C. The pulse labeling was then initiated by adding 15 μCi of [2-^{3}H]myoinositol (NEN-Dupont, 20 Ci/mmol) and incubation was continued for 20 min (lipid labeling experiments) or 60 min (protein labeling experiments) at 30°C with shaking. The incubation was stopped by adding NaF and NaN_3 to a final concentration of 10 mM and removing the cells to ice or a chase was initiated by adding 1× vitamin mix containing inositol (Dulić *et al.*, 1991). Chase was for 100 min and was used only for lipid labeling experiments. Strain RH401-7C was labeled with inositol for 40 min at 37°C. In some labelings 20 μg/ml myriocin (Sandoz AG; Horvath *et al.*, 1994) were added in all steps. The cells were then washed with cold water, collected by centrifugation, and resuspended in 400 μl water. For protein extractions (Horvath and Riezman, 1994), the cells were lysed by adding 40 μl of 2 M NaOH/5% β-mercaptoethanol and leaving on ice for 30 min. Proteins were then precipitated by adding 50 μl of cold 100% TCA. After another 30 min on ice, the precipitate was collected by centrifugation and the pellet was washed once with cold acetone. The dried pellet was resuspended, with the aid of sonication in a bath, and heated at 95°C in 120 μl of 2× sample buffer.

For extraction of labeled lipids (Horvath *et al.*, 1994), the washed cells were extracted by resuspending the cells in 500 μl $CHCl_3$-CH_3OH-H_2O (CMW, 10:10:3), and vortex mixing with glass beads as described above. The organic phase was saved and the glass beads were washed with 300 μl CMW. The organic phases were pooled and dried in the speedvac and extracted with butanol and water. The butanol phase was again dried and redissolved in 40 μl CMW. Separation then followed by TLC.

Labeling with [3-^{3}H]serine (NEN-Dupont, 29 Ci/mmol) was performed as the labeling with [^{3}H]inositol with following changes. The medium used for preincubation included inositol. For pulse labeling, 15 μCi of [^{3}H]serine was used and incubation time was prolonged to 30 min. Chase included addition of L-serine to a final concentration of 1 mM and lasted 120 min.

Membrane preparation

Cells were grown for membrane preparation (Zweerink *et al.*, 1992) on YPUD until they reached an OD_{600} of ~0.5, were harvested by centrifugation and washed with cold 50 mM phosphate buffer at pH 7.5. The cells were then resuspended in twice their volume of cold PDP (50 mM potassium phosphate pH 7.0, 5 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride) and broken by vortexing with 2.5 times the wet weight of the cell slurry of glass beads. Up to eight 1 min intervals of vortexing, with at least 1 min on ice in between, were necessary to break >80% of the cells. After allowing the beads to settle, the supernatant was removed, briefly centrifuged at low speed, and saved.

The glass beads were washed five times with 5 ml cold PDP. The pooled supernatants were spun at low speed (300 *g*) to remove large debris and unbroken cells and the membranes were then collected from the low speed supernatant by centrifugation for 30 min at 80 000 *g* at 4°C. The resulting pellet was washed twice by resuspending the membranes in PDP and centrifuging as above. Resuspending the pellet was facilitated by brief sonication. The washed membranes were then resuspended in a small volume of PDP containing 20% glycerol at a protein concentration of ~10 mg/ml.

Protein concentration measurements

Prior to analysis, membranes were diluted in 1% deoxycholate. Membrane proteins were then concentrated by precipitation with 10% TCA (Bensadoun and Weinstein, 1976). The pellet was washed with 5% TCA and resuspended in 0.1 N NaOH/1% SDS. Protein concentrations were measured (Peterson, 1977) using a standard curve that was prepared with bovine serum albumin.

Enzymatic assays

All reactions were carried out at 30°C and in a final volume of 100 µl. For assay of the synthesis of GlcN-acylPI (Costello and Orlean, 1992), membranes were preincubated for 5–10 min without radiolabel. The reaction mixture contained 100 mM Tris–HCl, pH 7.5, 1 mM EGTA, 3 mM Mg-acetate, 0.5 mM $MnCl_2$, 1 mM CoA, 1mM ATP, 20 µg/ml tunicamycin (Sandoz AG) and 400 µg membrane protein. The pre-incubated membranes were then transferred to a tube containing 15 µCi dried UDP-[^{3}H]GlcNAc (NEN-Dupont, 20–45 Ci/mmol) and incubated for 30 min at which time the reaction was stopped by addition of 1.5 ml $CHCl_3$-methanol (CM, 1:2). After thorough mixing and removal of insoluble components by centrifugation, the supernatant was saved and the pellet extracted a second time with 500 µl CM. The lipids from both extractions were pooled, dried and partitioned between butanol and water to remove salt. For analysis by TLC, the butanol phases were dried and resuspended in 20 µl CMW.

Serine-palmitoyltransferase was assayed (Zweerink *et al.*, 1992) in a buffer consisting of 100 mM HEPES, pH 8.3, 5 mM DTT, 2.5 mM EDTA, 50 µM pyridoxal-phosphate, 40 µM palmitoyl-CoA, 5 mM L-serine and 10 µCi [^{3}H]serine. Some reactions also contained 20 µg/ml myriocin. The reaction was initiated by adding 400 µg membrane protein and incubation was for 30 min. The reaction was terminated by addition of 20 µl 2 M NH_4OH. The lipids were extracted by adding 600 µl CM (1:2) and mixing thoroughly. Then 25 µg dihydrosphingosine (Sigma Chem. Co.) in 100% ethanol was added as a carrier, followed by 400 µl $CHCl_3$ and 800 µl 0.5 M NH_4OH and mixing. The upper aqueous phase was removed and the organic phase was washed four times with 600 µl water. The dried organic phase was finally redissolved in 20 µl CM (1:2) and analyzed by TLC.

Chemical and enzymatic treatment of lipids

To test the base sensitivity (Becker and Lester, 1980) of [^{3}H]myoinositol and [^{3}H]serine labeled lipids, 10 µl of lipids from the labeling experiments were incubated with 2 µl of 600 mM NaOH in 94% methanol for 30 min at 30°C. The reaction was neutralized by adding 2 µl of 1 M acetate. In some experiments stronger conditions were used for base hydrolysis by increasing the base to a final concentration of 200 mM and increasing incubation times for up to an hour.

For nitrous acid treatment, half the lipids of an *in vitro* labeling with UDP-[^{3}H]GlcNAc were used. The radiolabeled lipids were resuspended in 25 µl of 0.1 M NaAc, pH 3.5, 0.01% Zwittergent 3-16 (Calbiochem) and 25 µl of freshly prepared 0.5 M $NaNO_2$ or 0.5 M NaCl (control) was added. After 2 min of sonication in a waterbath the samples were incubated for 1 h at 60°C. This procedure was repeated five more times without sonication (Guther *et al.*, 1994).

For mild acid hydrolysis, UDP-[^{3}H]GlcNAc labeled lipids were resuspended in 20% (v/v) methanol and 20 mM HCl. After brief sonication the lipids were incubated for 20 min at 100°C and extracted twice with chloroform (Wilson *et al.*, 1993).

For PI-PLC digestion, UDP-[^{3}H]GlcNAc labeled lipids were resuspended in 50 µl of 20 mM Tris–HCl pH 7.5, 20% propanol, 1 mM EDTA and digested with or without 0.05 units PI-specific PLC (Boehringer, Switzerland) for 2 h at 30°C (Puoti and Conzelmann, 1992).

After chemical or enzymatic treatments, the solutions were extracted twice with water-saturated butanol. The combined butanol phases were washed twice with butanol-saturated water and dried in the Speedvac. The samples were dissolved in CMW and resolved by TLC.

Thin layer chromatography (TLC)

Analysis of lipids was performed routinely by TLC on plates with glass or aluminum backing (Silica 60; Merck) using a solvent system composed of $CHCl_3$-CH_3OH–25%NH_4OH–1 M (NH_4)acetate–H_2O (180:140:11:8:21). For the experiment in Figure 9, the solvent system was CMW. After migration, the plates were air dried and sprayed with En^{3}Hance (Dupont) and exposed to film at −70°C. Standards representing GlcNAc-PI and GlcN-PI were kindly provided by M.Ferguson (Dundee).

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