Mammalian PIG-L and its yeast homologue Gpi12p are *N*-acetylglucosaminylphosphatidylinositol de-N-acetylases essential in glycosylphosphatidylinositol biosynthesis

Reika WATANABE, Kazuhito OHISHI, Yusuke MAEDA, Nobuo NAKAMURA and Taroh KINOSHITA[1]

Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

Glycosylphosphatidylinositol (GPI) is used as a membrane anchor by many eukaryotic cell-surface proteins. The second step of GPI biosynthesis is de-N-acetylation of *N*-acetylglucosaminylphosphatidylinositol (GlcNAc-PI). We have previously cloned the rat PIG-L gene by expression cloning that complemented a mutant Chinese hamster ovary cell line defective in this step. Here we show that recombinant rat PIG-L protein purified from *Escherichia coli* as a complex with GroEL has GlcNAc-PI de-N-acetylase activity *in vitro*. The activity was not enhanced by GTP, which is known to enhance the de-N-acetylase activity of mammalian cell microsomes. As with other de-N-acetylases that act on the GlcNAc moiety, metal ions, in particular Mn^{2+} and Ni^{2+}, enhanced the enzyme activity of PIG-L. The *Saccharomyces cerevisiae* YMR281W open reading frame encodes a protein (termed Gpi12p) with 24% amino acid identity with rat PIG-L. On transfection into mammalian PIG-L-deficient cells, this gene, *GPI12*, restored the cell-surface expression of GPI-anchored proteins and GlcNAc-PI de-N-acetylase activity. The disruption of the gene caused lethality in *S. cerevisiae*. These results indicate that GlcNAc-PI de-N-acetylase is conserved between mammals and yeasts and that the de-N-acetylation step is also indispensable in yeasts.

Key words: endoplasmic reticulum, glycolipids, GroEL, metalloproteins, post-translational modification.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) is used to anchor various eukaryotic proteins to the cell-surface membrane [1,2]. GPI-anchored proteins are found in yeast [3], mammals [2] and plants [4] but are especially abundant in protozoan parasites [5]. The core structure and the biosynthetic steps of GPI are basically conserved in various organisms [5,6].

The common core structure of GPI consists of inositol phospholipid, GlcN, three mannoses and an ethanolamine phosphate [1]. The biosynthesis of GPI occurs on the membrane of the endoplasmic reticulum (ER) [7]. It is initiated by the transfer of GlcNAc to phosphatidylinositol from UDP-GlcNAc to generate *N*-acetylglucosaminylphosphatidylinositol (GlcNAc-PI). The second step is the de-N-acetylation of GlcNAc-PI to GlcN-PI. Later, three mannoses from dolichol-phosphate-mannose and an ethanolamine phosphate from phosphatidylethanolamine are added sequentially [7]. The core is variously modified with side groups during or after its synthesis [1]. Finally, an amino group of the terminal ethanolamine of the assembled GPI is linked to the C-terminus of a precursor protein [8]. This basic pathway of GPI biosynthesis was first investigated in trypanosomes [9]. A number of genes involved in GPI biosynthesis have been cloned in yeast and mammalian cells [2,10]; however, little is known about the characteristics of the enzymes of this biosynthesis.

The second-step enzyme, GlcNAc-PI de-N-acetylase, was partially purified from trypanosome membranes after solubilization with Zwittergent 3-14 [11]. The substrate specificity of de-N-acetylase was studied by using various GlcNAc-PI analogues [11–13]. However, this enzyme was so unstable in the presence of the detergent that purification was impossible [11]. It was also reported that de-N-acetylation of GlcNAc-PI in a mammalian cell-free system was stimulated by GTP hydrolysis [14]. The mechanism and a biological role of this GTP stimulation have yet to be clarified.

We have previously cloned a cDNA of the rat PIG-L gene that restores defective GlcNAc-PI de-N-acetylation in class L mutant cells after transfection [15]. Although PIG-L protein does not have a known ER retention signal in its sequence, it is localized in the ER membrane facing the cytosolic side of the membrane [15]. Moreover, overexpression of PIG-L resulted in the overexpression of GlcNAc-PI de-N-acetylase activity. These characteristics are consistent with the idea that PIG-L is GlcNAc-PI de-N-acetylase; however, because PIG-L has no similarity with proteins with known functions, its function could not be predicted.

Here we show that recombinant rat PIG-L protein expressed in and purified from *Escherichia coli* has GlcNAc-PI de-N-acetylase activity *in vitro*. We also identified human and *Saccharomyces cerevisiae* functional homologues of PIG-L that complemented class L mutant Chinese hamster ovary (CHO) cells. The yeast PIG-L homologue termed *GPI12* was essential for growth of the yeast, suggesting that it is the only gene that encodes GlcNAc-PI de-N-acetylase in yeast and that de-N-acetylation of GlcNAc-PI is essential for GPI synthesis in yeast, as it is in mammals and protozoa.

Abbreviations used: CHO, Chinese hamster ovary; DTT, dithiothreitol; ER, endoplasmic reticulum; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GlcNAc-PI, *N*-acetylglucosaminylphosphatidylinositol; GPI, glycosylphosphatidylinositol; Tos-Lys-CH_2Cl ('TLCK'), tosyl-lysylchloromethane.

[1] To whom correspondence should be addressed (e-mail tkinoshi@biken.osaka-u.ac.jp).

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AB017165 (human *PIG-L* cDNA) and AB017166 (*S. cerevisiae GPI12*).

© 1999 Biochemical Society

EXPERIMENTAL

Mammalian cells and plasmids

The CHO-K1 cell mutant M2S2, defective in the second step of GPI anchor biosynthesis, and its wild type IIIB2A (CHO-K1 cell transfected with human CD59 and DAF cDNA species) were as described previously [15]. They were cultured in Ham's F-12 medium supplemented with 10% (v/v) fetal-calf serum and 600 μg/ml geneticin. Human B lymphoblastoid JY25 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum, 0.2 mg/ml insulin and 0.2 mg/ml transferrin [16]. The mammalian expression plasmid containing Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG)-tagged rat PIG-L cDNA(pMEEB FLAG–PIG-L) was described previously [15]. To construct a plasmid containing FLAG-tagged GPI12, we amplified the *GPI12* gene from the genomic DNA of *S. cerevisiae* strain W303a/α [17] by PCR. To tag the N-terminus of Gpi12p with FLAG, we amplified a fragment of *GPI12* lacking an initiation codon but bearing a *Sal*I site at the 5′ end and an *Xba*I site at the 3′ end by using 5′ primer U1 (5′-GCTCGGGTCGACAAGATGTTGAGGCGTACAAAGGT-AA-3′) and 3′ primer L1 (5′-GCTCGGTCTAGACGGATCCT-CGATGACGATGAAGATGATGGTAT-3′). We then digested pMEEB FLAG–PIG-L with *Sal*I and *Xba*I to remove *PIG-L* and replaced it with a *Sal*I–*Xba*I fragment of the amplified *GPI12*. Plasmids (10 μg) were transfected into $(2–4)\times10^6$ cells suspended in 400 μl of culture medium without geneticin by electroporation at 260 V and 960 μF by using a Gene-Pulser (Bio-Rad). Transfectants were cultured in 200 μg/ml hygromycin to establish stable transfectants.

Expression in *E. coli* and purification of FLAG-tagged PIG-L

To construct pET–FLAG–PIG-L, we digested pMEEB FLAG–PIG-L and pET-19b (Novagen) with *Nco*I and *Bam*HI. The FLAG–PIG-L fragment from pMEEB FLAG–PIG-L was ligated with the digested pET-19b. *E. coli* BL21(DE3) transformed with pET-16b or pET–FLAG–PIG-L were grown in Luria–Bertani medium at 37 °C, induced by isopropyl β-D-thiogalactoside (0.5 mM) then subjected to an additional 3 h of culturing at 37 °C followed by centrifugation at 5000 ***g*** for 5 min and three washes with ice-cold PBS. The bacteria derived from 100 ml of culture were suspended in 10 ml of extraction buffer A [50 mM Tris/HCl (pH 8.0)/5 mM EDTA/50 μg/ml NaN_3/1 μg/ml leupeptin/0.1 mM tosyl-lysylchloromethane (Tos-Lys-CH_2Cl, 'TLCK')], incubated with lysozyme (0.25 mg/ml at room temperature for 5 min), mixed with 1 ml of extraction buffer B (1.5 M NaCl/0.1 M $CaCl_2$/0.1 M $MgCl_2$/0.02 mg/ml DNase I/0.05 mg/ml ovomucoid protease inhibitor) and incubated at room temperature for 5 min. The suspension was sonicated at 70 W for 70 s. To dissociate the fusion proteins partly from GroEL, the sonicated suspension was incubated with 10 mM $MgSO_4$/2 mM ATP/0.5 mM dithiothreitol (DTT) at 37 °C for 10 min. Alternatively, when the effects of metals and chelating reagent were being studied, the bacteria were suspended in PBS (without Mg^{2+} and Ca^{2+}) containing 1 μg/ml leupeptin and 0.1 mM Tos-Lys-CH_2Cl, then sonicated. The insoluble materials were removed by centrifugation at 100000 ***g*** for 1 h at 4 °C. The FLAG-tagged proteins were captured from the supernatant with 50 μl of anti-FLAG M2 affinity gel (Eastman Kodak, Rochester, NY, U.S.A.) and eluted with 100 μl of 0.8 mM FLAG peptide in 50 mM Hepes/NaOH, pH 7.4, containing 25 mM KCl, 0.1 mM Tos-Lys-CH_2Cl and 1 μg/ml leupeptin.

Western blotting and FACS analysis

CHO cells (10^5) and *E. coli* (equivalent to 0.1 μl of culture), both expressing FLAG-tagged PIG-L, were solubilized with SDS/PAGE sample buffer and were separated by SDS/PAGE [10% (w/v) gel], transferred to PVDF membrane and subjected to Western blot analysis with biotinylated anti-FLAG monoclonal antibody M2 plus horseradish peroxidase–streptavidin conjugates (Amersham) and detected by chemiluminescence (Renaissance; DuPont). FACS analysis was performed as described previously [18].

Preparation of cell lysates and biosynthesis of GPI precursors *in vitro*

The lysates of JY25 and CHO cells were prepared as described previously [19]. They were incubated with UDP-[6-^{3}H]GlcNAc (13.3 μCi/ml) for 3 h at 37 °C in a buffer consisting of 50 mM Hepes/NaOH, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM Tos-Lys-CH_2Cl, 1 μg/ml leupeptin, 5 mM $MnCl_2$, 1 mM ATP, 0.5 mM DTT and 0.2 μg/ml tunicamycin. The lipids were extracted with $CHCl_3$/CH_3OH/water (10:10:3, by vol.). The lipid extract was dried, dissolved in water-saturated butan-1-ol and partitioned with water. The butanol fraction was dried. The glycolipids were analysed by TLC with the solvent system $CHCl_3$/CH_3OH/1 M NH_4OH (10:10:3, by vol.) followed by image analysis [20]. To quantify the GlcNAc-PI and GlcN-PI generated, the intensities of the spots were measured with a Fuji Image Analyser BAS 1500 (Fuji Film Co., Tokyo, Japan) after exposure for 2–5 days.

Acetylation of [6-^{3}H]GlcN-PI to generate [6-^{3}H]GlcNAc-PI

Lysates of JY25 cells equivalent to 4×10^8 cells were incubated with 80 μCi of UDP-[6-^{3}H]GlcNAc (13.3 μCi/ml) for 3 h at 37 °C as described above. Glycolipids extracted by butan-1-ol and dried were dissolved in 2.8 ml of methanol and then mixed with 2.8 ml of $NaHCO_3$-saturated water. The addition of acetic anhydride (280 μl) was followed by incubation on ice for 10 min. A further 280 μl of acetic anhydride was added and the mixture was incubated at room temperature for 50 min. GlcNAc-PI was extracted as described above and a small aliquot was used to measure radioactivity [21].

Assay of GlcNAc-PI de-N-acetylase

[6-^{3}H]GlcNAc-PI synthesized *in vitro* and dissolved in 1 μl of 99% (v/v) ethanol was incubated with enzyme samples with or without 1 mM GTP in 150 μl of a buffer consisting of 50 mM Hepes/NaOH, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM DTT, 0.1 mM Tos-Lys-CH_2Cl and 1 μg/ml leupeptin at 37 °C for 0–120 min. When studying the effect of metal ions and chelating reagent, we used 50 mM Hepes/NaOH (pH 7.4)/25 mM KCl/0.5 mM DTT/0.1 mM Tos-Lys-CH_2Cl/1 μg/ml leupeptin as a basic buffer. The deacetylated products were extracted and analysed as described above.

Cloning of human *PIG-L* cDNA

We searched an expressed sequence tag database [22] for a human homologue of rat *PIG-L*. We purchased a plasmid of a clone 565114 from A. T. C. C. (Rockville, MD, U.S.A.). To obtain a sequence of the 5′ end of human *PIG-L*, we amplified it from human placenta cDNA (Clontech) by PCR. We used two 5′ primers AP1 and AP2, corresponding to the Marathon cDNA adaptor (Clontech), and two human *PIG-L*-specific 3′ primers,

© 1999 Biochemical Society

hPIG-L R1 (5′-ATGAAGCAGAGACAAGGGCAGAT-3′) and hPIG-L R2 (5′-CAGATTGATGCCATTCACTTCTA-3′), designed from the sequence of clone 565114. We amplified the 5′ fragment of human *PIG-L* by nested PCR first with primers AP1 and hPIG-L R1, and then with primers AP2 and hPIG-L R2. The product was subcloned into *Eco*RV-digested pBluescript II (Stratagene, La Jolla, CA, U.S.A.) and sequenced. To obtain a full-length human *PIG-L* cDNA, we ligated the 5′ fragment of human *PIG-L* from the PCR product and the remainder of the 3′ fragment from clone 565114 at the *Sph*I site. This human *PIG-L* cDNA was subcloned into a mammalian expression plasmid pME-Pyori18Sf− [23] and transfected into M2S2 CHO cells to confirm its function.

Yeast strains and growth conditions

S. cerevisiae diploid strain W303a/α (*ura3-52 trp1-1 ade2-1 lys2-801 leu2-3 his3-11, 15 can1-100*) was used [17]. Diploid yeasts were left to sporulate on sporulation medium at 30 °C for 3–5 days. Tetrads were dissected on yeast extract/peptone/dextrose (YPD) plates and incubated at 30 °C for 2–3 days to allow the spores to germinate. Ura^+ transformants were negatively selected on supplemented minimal medium (SMM) plates containing uracil and 0.5 mg/ml 5-fluoro-orotic acid. These yeast media were prepared as described previously [24].

Disruption of *GPI12* gene

We amplified the 2.5 kbp genomic fragment containing the *GPI12* gene, introducing a *Not*I site at the 5′ end and a *Sal*I site at the 3′ end by PCR with the 5′ primer U2 (5′-GCTCGGCGGGCGGCCGCCTGGGTTCCAAACCTTG-TCGATCAG-3′) and the 3′ primer L2 (5′-GCTCGGGTC-GACGGCATTTAATGAAGCAGTGGTATGCA-3′). The fragment and pBluescript (pBS) II were digested with *Not*I and *Sal*I and ligated to generate pBS GPI12. To prepare a knock-out construct of *GPI12,* we digested pBS GPI12 with *Xba*I and *Hin*dIII, and YDp-L [25] with *Bam*HI. The 4.5 kbp fragment of pBS GPI12 lacking the *Hin*dIII and *Xba*I fragment of *GPI12* gene, and the 1.6 kbp *Bam*HI fragment containing the yeast *LEU2* gene excised from YDp-L, were treated with T4 DNA polymerase, ligated and then cut at *Pst*I (in *GPI12*) and *Sal*I (in pBS) sites. This 2.5 kbp *GPI12* knock-out construct was used to transform yeast strain W303a/α [26]. Leu^+ transformants were selected and disruption of the *GPI12* gene was confirmed by Southern blotting. The 0.65 kbp fragment excised from pBS GPI12 with *Not*I in pBS and *Pst*I was used as a probe.

To construct multi-copy plasmids containing the wild-type *GPI12* gene (YEplac GPI12), we digested pBS GPI12 and YEplac195 [27] with *Sac*I and *Sal*I. The 2.5 kbp fragment containing the *GPI12* gene and digested YEplac195 were ligated.

RESULTS

Expression and purification of rat PIG-L protein

We previously cloned a cDNA of rat PIG-L, a candidate GlcNAc-PI de-N-acetylase that catalyses the second step of GPI biosynthesis [15]. To determine whether PIG-L is the de-N-acetylase itself, we tagged its N-terminus with FLAG peptide and expressed it in CHO cells or in *E. coli*. Immunoblot analysis with anti-FLAG antibodies (Figure 1) revealed that both FLAG-tagged PIG-Ls expressed in CHO cells (lane 2) and in *E. coli* (lane 4) had an apparent molecular mass of 32 kDa, slightly more than that (29 kDa) predicted from the amino acid sequence. The three higher-molecular-mass bands seen in Figure 1 (lane 2) should be non-specific because they were also seen in non-transfected CHO cells (lane 1). We then affinity-purified recombinant FLAG-tagged PIG-L expressed in *E. coli* with anti-FLAG beads. Silver staining of the affinity-purified FLAG-tagged rat PIG-L (Figure 1, lane 5) showed that it co-purified with 60 kDa protein. N-terminal amino acid sequencing identified it as GroEL, a molecular chaperone that binds to the hydrophobic surface of the non-native portion of proteins [28]. Rat PIG-L protein has a hydrophobic region near the N-terminus that might act as an anchor to the ER membrane [15]. GroEL might have bound to this region, preventing aggregation.

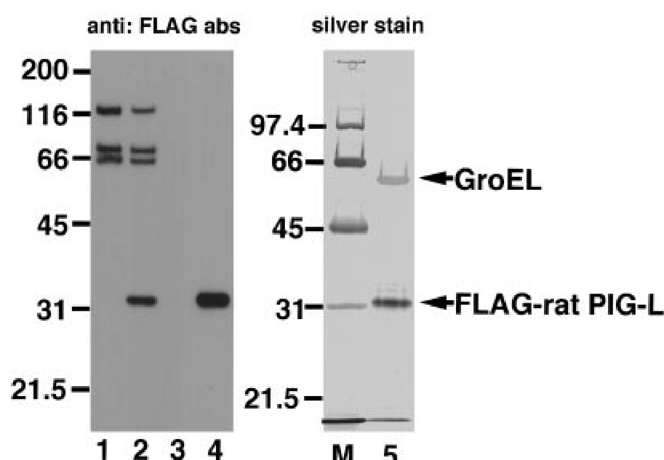

Figure 1 FLAG-tagged PIG-L expressed in CHO cells and *E. coli*

Lanes 1–4, Western blot analysis of FLAG-tagged PIG-L expressed in CHO cells or *E. coli*. CHO cells (10^5) transfected with pMEEB FLAG–PIG-L (lane 2) or not (lane 1), and *E. coli* (equivalent to 0.1 μl of culture) transformed with pET FLAG–PIG-L (lane 4) or pET vector (lane 3), were solubilized with SDS sample buffer and analysed by Western blotting with anti-FLAG antibodies. The three higher-molecular-mass bands in lanes 1 and 2 should be non-specific. Lane 5, silver staining of FLAG-tagged PIG-L affinity-purified from *E. coli* using anti-FLAG beads. The positions of molecular mass markers (lane M) are indicated (in kDa) at the left. The identities of bands are also indicated.

GlcNAc-PI de-N-acetylase activity of recombinant PIG-L protein

We assessed the enzymic activity of purified PIG-L protein *in vitro*. As shown in Figure 2(A), radiolabelled GlcNAc-PI (approx. 3000 d.p.m.) was incubated with purified recombinant PIG-L protein (three-fifths of the amount shown in Figure 1, lane 5) at 37 °C for the indicated periods (Figure 2A, lanes 2–7). We measured the intensities of the spots and calculated the percentage of deacetylated product GlcN-PI in total radiolabelled glycolipids. As shown in Figure 2(B), after incubation with recombinant PIG-L/GroEL protein complex, the percentage of GlcN-PI increased with time and reached 70 % at 120 min. No significant amount of GlcN-PI was seen after 120 min of incubation with proteins that were mock affinity-purified from *E. coli* transformed with an empty vector (Figure 2A, lane 1). This result indicated that PIG-L protein has GlcNAc-PI de-N-acetylase activity.

Effect of GTP on recombinant GlcNAc-PI de-N-acetylase

It has been reported that the incubation of microsomes with GTP enhanced the GlcNAc-PI de-N-acetylase activity *in vitro*

© 1999 Biochemical Society

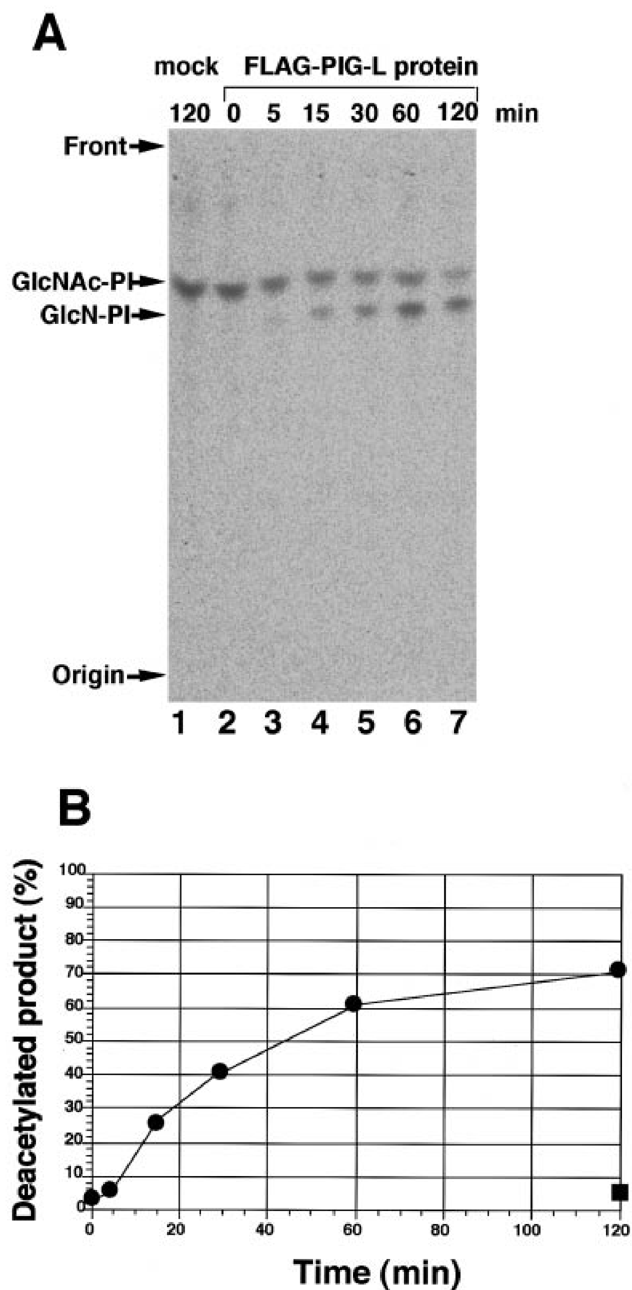

Figure 2 GlcNAc-PI de-N-acetylase activity of recombinant FLAG–PIG-L proteins *in vitro*

(**A**) Radiolabelled GlcNAc-PI synthesized *in vitro* (3000 d.p.m. per lane) was incubated at 37 °C with mock-affinity-purified proteins (lane 1) or affinity-purified recombinant FLAG–PIG-L protein (three-fifths of the amount shown in Figure 1, lane 5) for the indicated periods (lanes 2–7). Radiolabelled glycolipids were analysed by TLC. The identities of the glycolipid spots are indicated. (**B**) The amounts of radioactivity in the spots of GlcNAc-PI and GlcN-PI were quantified and the percentages of deacetylated products (GlcN-PI) in total radiolabelled glycolipids were determined. Symbols, ■, mock; ●, FLAG–PIG-L.

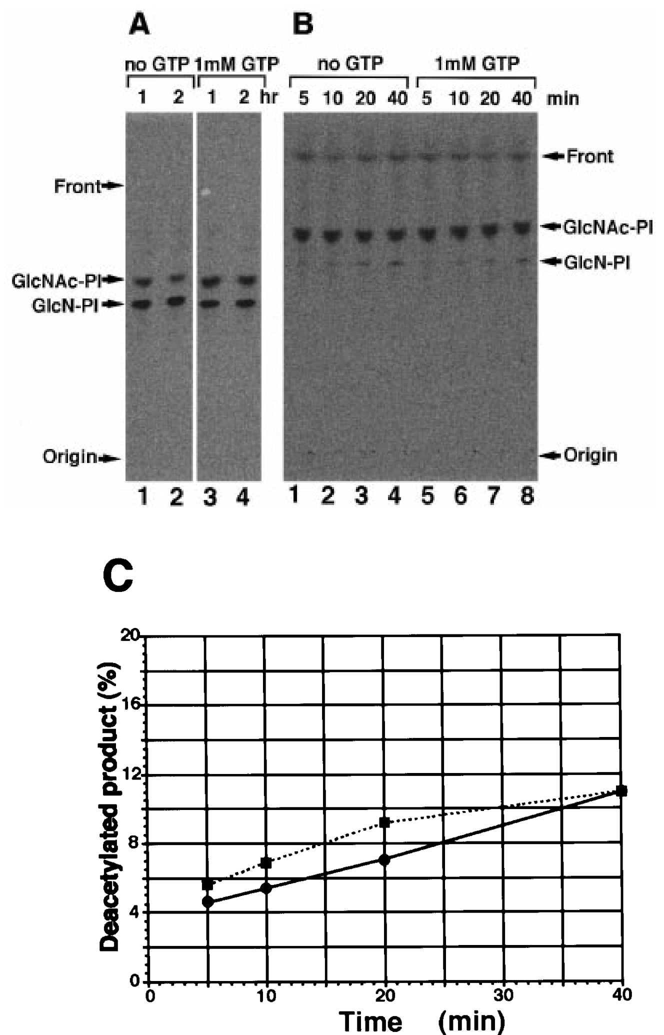

Figure 3 Effects of GTP on recombinant GlcNAc-PI de-N-acetylase

(**A**) Recombinant PIG-L was preincubated with GTP (lanes 3 and 4) or without GTP (lanes 1 and 2) for 5 min at 37 °C. Radiolabelled GlcNAc-PI was added and the mixture was incubated at 37 °C for 1 h (lanes 1 and 3) or 2 h (lanes 2 and 4). (**B**) Recombinant PIG-L was incubated with GTP (lanes 5–8) or without GTP (lanes 1–4) for 5 min (lanes 1 and 5), 10 min (lanes 2 and 6), 20 min (lanes 3 and 7) and 40 min (lanes 4 and 8). Radiolabelled glycolipids were analysed by TLC. The identities of the glycolipid spots are indicated. (**C**) The amounts of radioactivity in the spots of GlcNAc-PI and GlcN-PI identified in (**B**) were quantified and the percentages of deacetylated products (GlcN-PI) in total radiolabelled glycolipids were calculated. Solid line (●), 1 mM GTP; dotted line (■), no GTP.

[14]. The effects of GTP on the de-N-acetylase of membrane were twofold. First, the presence of 1 mM GTP increased the total amount of deacetylated products approx. 2-fold. Secondly, in the presence of GTP, the deacetylation reaction seemed to be biphasic with an initial fast phase and a subsequent slower phase. The fast phase in the presence of GTP was accelerated approx. 5-fold compared with the reaction in the absence of GTP [14]. We examined whether the de-N-acetylase activity of recombinant PIG-L protein was enhanced by GTP. The recombinant proteins were preincubated with 1 mM GTP, then substrate was added and incubated for 1 or 2 h (Figure 3A). The amounts of deacetylated products were similar with or without GTP. We next tested shorter incubation durations to see the effect of GTP during an initial phase. The percentage of de-N-acetylated product increased similarly with or without GTP (Figures 3B and 3C). We conclude that GTP does not directly enhance the enzymic activity of PIG-L protein.

© 1999 Biochemical Society

Table 1 Effects of metal ions and EDTA on de-N-acetylation activity of PIG-L and JY25 lysates

Samples were prepared without metal ions and preincubated with or without 5 mM EDTA for 5 min at 37 °C; radiolabelled GlcNAc-PI was then added and the mixture was incubated for 2 h (recombinant protein) or 1 h (cell lysates). Activities are normalized to those in the absence of EDTA and metal ions. For the incubations without EDTA, recombinant PIG-L protein bound to anti-FLAG beads was pretreated with 5 mM EDTA for 5 min; then, after being washed three times with a buffer, PIG-L was eluted and used.

	Relative activity														
	Recombinant PIG-L protein										Cell lysates				
Treatment	EDTA only	Mn^{2+}	Ni^{2+}	Ca^{2+}	Mg^{2+}	Co^{2+}	Cu^{2+}	Fe^{2+}	Fe^{3+}	Zn^{2+}	EDTA only	Mn^{2+}	Ca^{2+}	Ni^{2+}	Mg^{2+}
EDTA + 10 mM metal	1.0	4.6	8.0	3.1	2.7	1.6	1.1	1.7	1.3	0.6	0.7	6.0	4.2	2.0	0.7
No EDTA + 2 μM metal	–	1.1	1.0	0.9	1.0										
No EDTA + 100 μM metal	–	1.7	1.7	1.3	1.2										

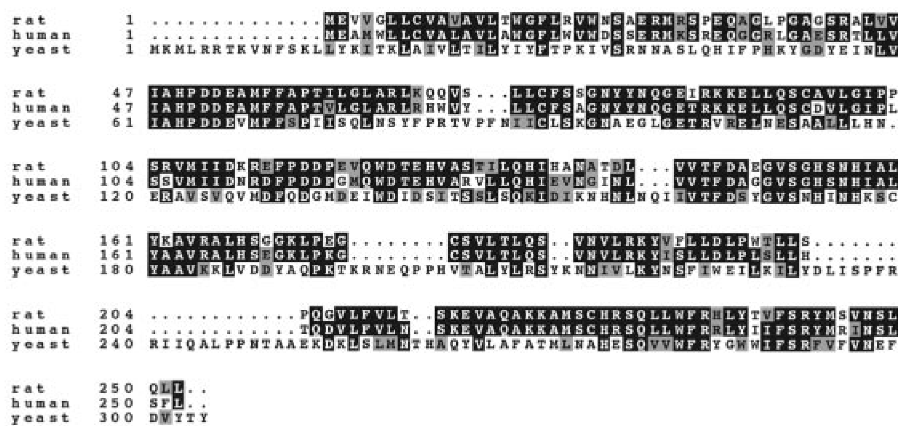

Figure 4 Alignment of the rat and human PIG-L and *S. cerevisiae* Gpi12p sequences

Identical and similar amino acids are shown in black and shaded backgrounds respectively.

Effects of metal ions and EDTA

Some de-N-acetylases that react with GlcNAc moiety such as microsomal *N*-acetylglucosaminyl de-N-acetylase (involved in heparin biosynthesis) [29], *N*-acetylglucosamine-6-phosphate de-N-acetylase [30] and UDP-3-*O*-acyl-GlcNAc de-N-acetylase [31] are metalloproteins. In addition, the activity of GlcNAc-PI de-N-acetylase in the microsome fraction was enhanced by Mg^{2+} ions [7]. We examined the effects of various metal ions and EDTA on PIG-L protein isolated from *E. coli* (Table 1). We tested $MnCl_2$, $NiCl_2$, $CaCl_2$, $MgCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$ and $ZnCl_2$ at 10 mM in the presence of 5 mM EDTA. First we found that the de-N-acetylase activity of recombinant PIG-L protein was not affected by 5 mM EDTA. $MnCl_2$ and $NiCl_2$ enhanced the de-N-acetylase activity 4.6-fold and 8-fold respectively; $CaCl_2$ and $MgCl_2$ enhanced it approx. 3-fold. Other metal ions had no significant effect. Next we assessed the effect of metal ions at lower concentrations (2–100 μM) after pretreatment of PIG-L protein with EDTA. $MnCl_2$ and $NiCl_2$ at 100 μM enhanced the activity less than 2-fold (1.7-fold). $CaCl_2$ and $MgCl_2$ at 100 μM enhanced it slightly. At 2 μM, no metal ions had a significant effect. We also assessed the effect of metal ions on de-N-acetylase activity in lysates of JY25 cells in a similar way. The activity was decreased to 70 % with 5 mM EDTA. $MnCl_2$, $CaCl_2$ and $NiCl_2$, each at 10 mM in the presence of 5 mM EDTA, enhanced the de-N-acetylase activity approx. 6-fold, 4-fold and 2-fold respectively. $MgCl_2$ did not enhance the activity.

Complementation of hamster class L mutant cell line with human *PIG-L* and yeast *GPI12*

A Basic Local Alignment Search Tool (BLAST) search of the databases revealed the presence of human expressed sequence tag clones and *S. cerevisiae* open reading frame that had high similarity scores with the rat PIG-L amino acid sequence [15]. We obtained a 1.1 kb cDNA of putative human PIG-L (see the Experimental section) encoding a 252-residue protein like rat PIG-L that had 77 % amino acid identity with rat PIG-L protein (Figure 4). A *S. cerevisiae* YMR281W open reading frame encodes a putative 304-residue protein with 24 % amino acid identity with rat PIG-L (Figure 4). The hydropathy profiles of the three proteins are similar overall (results not shown).

To determine whether these structural human and yeast homologues of rat PIG-L were functional PIG-L homologues, we cloned them into a mammalian expression vector and transfected them into class L mutant CHO cells that stably expressed human CD59 precursor peptide as a marker GPI-anchored protein. A human PIG-L homologue restored the surface expression of CD59 on the class L mutant to the same level as those in wild-type IIIB2A CHO cells and mutant CHO cells transfected with rat PIG-L cDNA (results not shown). We

© 1999 Biochemical Society

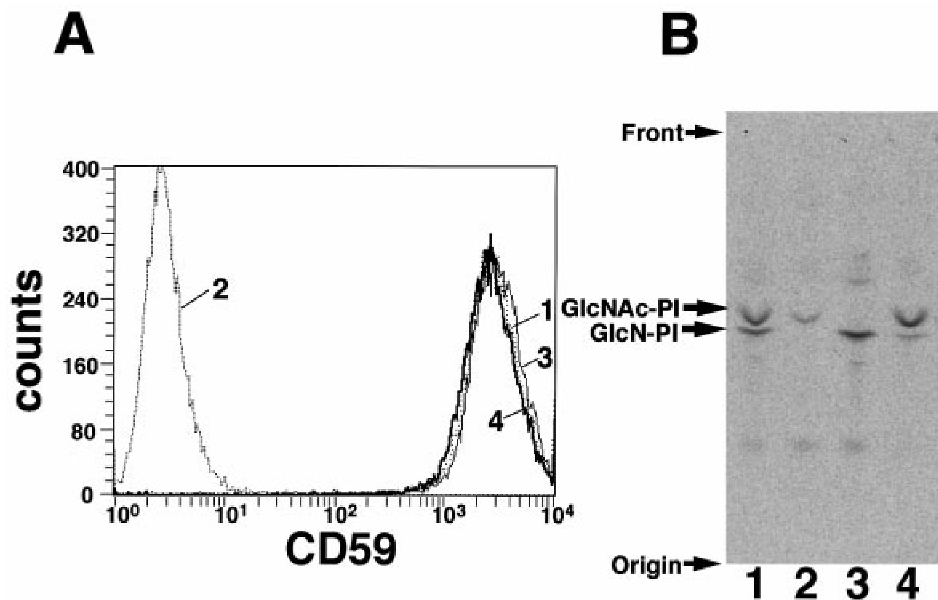

Figure 5 Complementation of class L mutant cells with *S. cerevisiae GPI12*

(**A**) Restoration of the surface expression of GPI-anchored proteins (CD59) on class L mutant cells with *S. cerevisiae GPI12*. Curve 1, wild-type IIIB2A; curve 2, class L mutant M2S2; curve 3, M2S2 cells transfected with FLAG-tagged rat PIG-L cDNA; curve 4, M2S2 cells transfected with FLAG-tagged *S. cerevisiae* GPI12 DNA. (**B**) Restoration of GlcNAc-PI de-N-acetylase activity of class L mutant cells by transfection with *S. cerevisiae GPI12*. Cell lysates from wild-type IIIB2A (lane 1), M2S2 mutant (lane 2), M2S2 cells transfected with FLAG-tagged rat PIG-L (lane 3) and M2S2 cells transfected with FLAG-tagged *S. cerevisiae* GPI12 (lane 4) were incubated with UDP-[6-^{3}H]GlcNAc. The extracted glycolipids were analysed by TLC and the percentages of deacetylated products (GlcN-PI) in total radiolabelled glycolipids were calculated.

concluded that this cDNA encodes functional human PIG-L protein. The yeast homologue also restored the surface expression of CD59 on PIG-L mutant cells (Figure 5A, curve 4). We further assessed the restoration of de-N-acetylase activity by the yeast PIG-L homologue system *in vitro* by using UDP-[6-^{3}H]GlcNAc and cell lysate (Figure 5B). Class L cells were defective in the generation of GlcN-PI as expected (Figure 5B, lane 2). After transfection with a plasmid encoding yeast PIG-L homologue tagged with FLAG at the N-terminus, GlcN-PI was synthesized (Figure 5B, lane 4), although the amount was much smaller than in wild-type cells (lane 1) and in the mutant cells transfected with FLAG-rat PIG-L (lane 3). The percentage of GlcN-PI in the total of GlcNAc-PI and GlcN-PI was estimated to be 41 % in wild-type cells (Figure 5B, lane 1), 1.8 % in mutant cells (lane 2), 98 % in mutant cells expressing rat PIG-L (lane 3) and 11 % in mutant cells expressing yeast PIG-L (lane 4). The different de-N-acetylase activities between the latter two cells might have been due to a much lower expression of yeast PIG-L than of rat PIG-L because yeast PIG-L protein was undetectable with Western blotting with the use of anti-FLAG, whereas rat PIG-L was easily detected (results not shown). In addition, the synthesis of GlcNAc-PI from UDP-[6-^{3}H]GlcNAc was suppressed in the mutant cells (Figure 5B, lane 2), suggesting that the accumulated endogenous GlcNAc-PI inhibited the new incorporation of labelled GlcNAc into phosphatidylinositol. In mutant cells expressing yeast PIG-L (Figure 5B, lane 4), the total amount of radiolabelled GlcNAc-PI and GlcN-PI was restored to that in the wild type. We concluded that YMR281W is a functional homologue of mammalian PIG-L and named the gene *GPI12*.

Essential nature of *GPI12*

In *S. cerevisiae*, the GPI anchor is essential for viability [32,33]. To test whether *GPI12* is an essential gene, we disrupted it with the *LEU2* gene (Figure 6A). To confirm that the disrupted copy of *GPI12* had been integrated at the expected chromosomal locus in the diploid strain, we isolated genomic DNA from *LEU2*$^+$ transformants and digested it with *Cla*I or *Eco*47III. A Southern blot analysis performed with the probe indicated in Figure 6(A) demonstrated that the expected fragments were obtained, showing that diploid transformants harboured one intact gene (*GPI12*) and one disrupted copy (*gpi12::LEU2*) (Figure 6B). The heterozygous *GPI12/gpi12::LEU2* yeast was allowed to sporulate. On tetrad dissection, only two spores from each tetrad could grow (Figure 6C). These two spores required leucine for growth, indicating that they must contain the intact *GPI12* gene, whereas the two non-viable spores received the *LEU2* gene. To discern whether the cloned *GPI12* gene could rescue non-viable *gpi12::LEU2* haploids, we transformed the *GPI12/gpi12::LEU2* diploid with a multicopy plasmid containing the wild-type *GPI12* gene and the *URA3* gene as a marker and allowed it to sporulate. More than half of the tetrads analysed contained four viable spores, of which two required leucine for growth (*GPI12*) and two did not (*gpi12::LEU2*) (results not shown). All mutant (*gpi12::LEU2*) haploids were sensitive to 5-fluoro-orotic acid, which is toxic to cells containing the plasmid-borne *URA3* marker (results not shown). These results showed that *GPI12* is essential for the viability of *S. cerevisiae*. The essentiality of this gene indicates that there is no other functional equivalent of GlcNAc-PI de-N-acetylase in *S. cerevisiae*.

DISCUSSION

In this study we showed that rat PIG-L protein has GlcNAc-PI de-N-acetylase activity. We also reported its human and yeast functional homologues, which complement class L mutant CHO cells. They share 77 % and 24 % amino acid identity with rat PIG-L protein respectively. The yeast homologue, termed *GPI12*,

© 1999 Biochemical Society

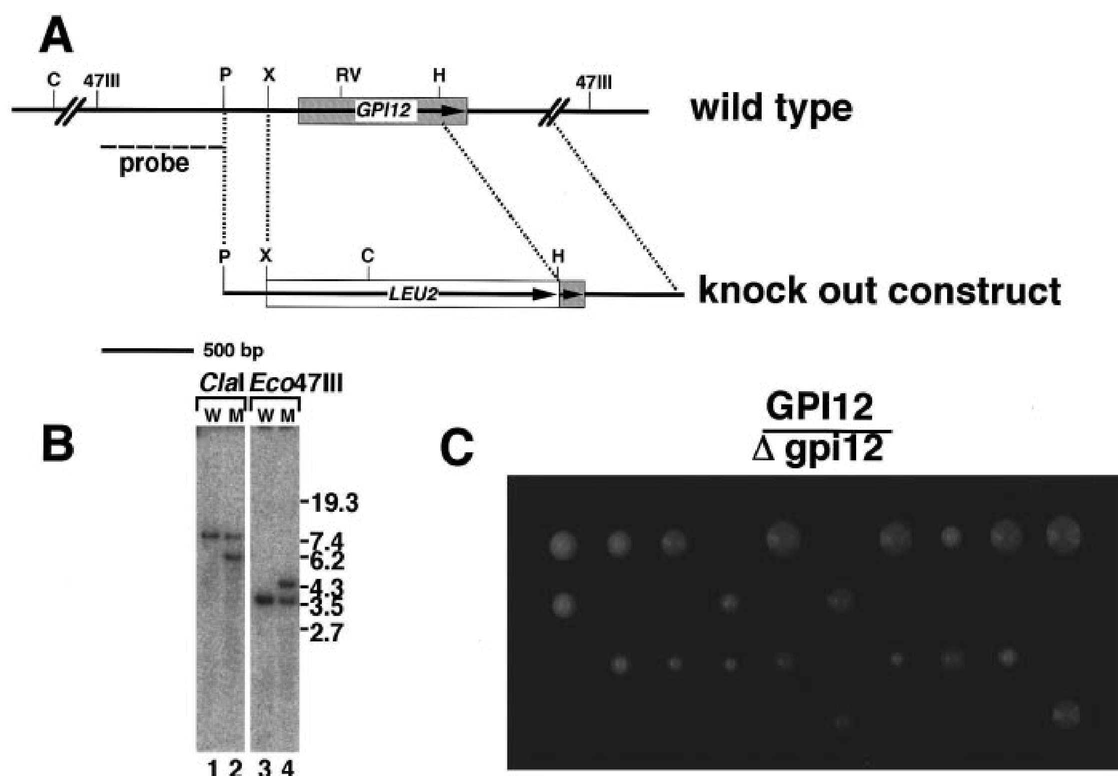

Figure 6 Disruption of the *GPI12* gene

(**A**) Restriction maps showing how the *GPI12* gene was disrupted. The 1.6 kbp *Bam*HI fragment containing yeast *LEU2* gene was used to replace 927 bp *Xba*I and *Hin*dIII fragments of the *GPI12* gene. Restriction endonuclease sites are indicated as follows: P, *Pst*I; X, *Xba*I; RV, *Eco*RV; H, *Hin*dIII; C, *Cla*I; 47III, *Eco*47III. (**B**) Southern blot analysis of genomic DNA from wild-type (W) and *GPI12*/*gpi12::LEU2* (M) diploid strains. The 0.65 kbp fragment indicated in (**A**) was used as a probe. The predicted sizes of the bands yielded by the *Cla*I digest are 7.7 kb (wild-type) and 5.6 kb (*gpi12::LEU2* disruption), and those resulting from the *Eco*47III digest are 3.6 kb (wild-type) and 4.3 kb (*gpi12::LEU2* disruption). The positions of molecular mass markers are indicated (in kbp) at the right. (**C**) Tetrad analysis of *GPI12*/*gpi12::LEU2* diploid strains. After tetrad dissection, only two spores from each tetrad grew.

was essential for growth, suggesting that it is the only gene that encodes GlcNAc-PI de-N-acetylase in *S. cerevisiae* and that de-N-acetylation of GlcNAc-PI is an essential step in GPI biosynthesis in yeast, as it is in mammals [34] and trypanosomes [12,35].

We have demonstrated previously that PIG-L protein is localized in the ER, facing the cytosol [15]. The fact that PIG-L is the de-N-acetylase itself confirms that de-N-acetylation occurs on the cytoplasmic side of the ER. This is consistent with the membrane orientation of GlcN-PI [36]. Taken together with previous reports that both the first-step enzyme [18] and GlcNAc-PI [36] are cytoplasmically oriented, it is now clear that the first two reactions proceed on the cytoplasmic side of the ER.

Another de-N-acetylation mutant, class J, has been reported [37]. Although complementation analysis has not been done with the class L mutant, it is likely that the gene defective in class J is also *PIG-L* because PIG-L alone would be sufficient to mediate the second step. Alternatively, because the PIG-L protein has no typical ER retention signal, the hypothetical PIG-J protein might be involved in the ER localization of PIG-L.

A major fraction of FLAG-tagged rat PIG-L proteins expressed in *E. coli* was included in insoluble inclusion bodies and only a minor fraction was soluble. We captured soluble FLAG-tagged PIG-L protein with anti-FLAG beads in the absence of detergent. This recombinant FLAG–PIG-L was co-purified with GroEL, a molecular chaperone that binds to the hydrophobic surface of protein [28]. It is known that recombinant proteins are sometimes co-purified with GroEL [38,39]. GroEL is a large homotetradecamer composed of two seven-membered rings of 57 kDa subunits associating back to back. It is conceivable that the cylindrical complex of GroEL binds to the N-terminal hydrophobic region of PIG-L protein that might be involved in the enzyme's association with the ER membrane within the intact cells. The rest of the PIG-L molecule, which is essentially hydrophilic, might have a catalytic site. This portion should have folded properly within *E. coli* because a complex of PIG-L and GroEL showed GlcNAc-PI de-N-acetylase activity.

It was reported that GTP specifically enhanced the GlcNAc-PI de-N-acetylase activity of microsomes *in vitro* [14]. PIG-L protein does not have a typical GTP-binding motif [15]. In our experiments, the GlcNAc-PI de-N-acetylase activity of recombinant PIG-L protein was not enhanced by GTP (Figure 3). These results together suggest that GTP does not bind to PIG-L and that it does not enhance de-N-acetylase activity directly but does so indirectly. For example, GTP might enhance the accessibility of the substrate synthesized by the first enzyme to GlcNAc-PI de-N-acetylase [14]. We reported that the first enzyme complex and PIG-L exist separately in a digitonin extract [20]. If these two enzymes exist on separate regions of the ER membrane *in vivo*, they might exist on separate microsomal vesicles prepared by means of hypotonic disruption. GTP might be involved in the transfer of GlcNAc-PI from one vesicle to another, enhancing the accessibility of GlcNAc-PI to the de-N-acetylase. However, at present, the possibility cannot be ruled out that GroEL that

© 1999 Biochemical Society

bound to PIG-L might have influenced the effect of GTP on PIG-L protein.

Using purified PIG-L protein, we found that some metal ions enhanced GlcNAc-PI de-N-acetylase activity 3–8-fold at millimolar concentrations. In contrast, the de-N-acetylase activity was not inhibited by 5 mM EDTA. It is not clear whether recombinant PIG-L protein contains some metal ions essential for catalytic activity because in some enzymes metal ions are so tightly bound to the enzyme that treatment with EDTA was not efficient in removing metal ions [40,41]. Alternatively, metal ions might function to stabilize the conformation of the enzyme or increase its affinity for the substrate [41,42]. The enhancement of the activity by metal ions is not specific to recombinant PIG-L protein expressed in *E. coli* because it was also seen when mammalian cell lysates were used. Metal ions at micromolar concentrations had no significant effect. It is therefore uncertain whether these metals have a role in the enzymic activity under physiological conditions.

GPI12, which was identified on the basis of sequence similarity, complemented the mammalian PIG-L-deficient cells, suggesting that GlcNAc-PI de-N-acetylase is widely conserved among eukaryotes. GlcNAc-PI de-N-acetylases of other species might be identified on the basis of sequence similarity or by expression cloning with GlcNAc-PI de-N-acetylase-deficient cells. In the trypanosome cell-free system, the upstream intermediate GlcNAc-PI was an approx. 6-fold better substrate than GlcN-PI for the later mannosyltransferase [35]. Because the de-N-acetylation of GlcNAc-PI must precede mannosylation in the GPI biosynthetic pathway [12], this suggests a degree of substrate channelling via the GlcNAc-PI de-N-acetylase to the mannosyltransferase, indicating complex formation between the two enzymes. The identification of a trypanosomal homologue of GlcNAc-PI de-N-acetylase might lead to the identification of the subsequent GlcN-PI mannosyltransferase.

The present study is the first report of the identification of GlcNAc-PI de-N-acetylase in mammals and yeast. The purified recombinant PIG-L protein will facilitate the determination of active-site residues and the substrate specificity of this enzyme. Yeast de-N-acetylase can rescue a mammalian de-N-acetylation mutant. It is possible that mammalian PIG-L also complements yeast de-N-acetylation mutant. If so, we can use yeast cells expressing yeast or mammalian GlcNAc-PI de-N-acetylase to screen the species-specific inhibitors.

We thank Dr. Hiroyuki Araki and Dr. Yoichiro Kamimura for precise advice on yeast studies and for providing us with yeast strains and vectors. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1 Ferguson, M. A. and Williams, A. F. (1988) Annu. Rev. Biochem. **57**, 285–320
2 Kinoshita, T., Inoue, N. and Takeda, J. (1995) Adv. Immunol. **60**, 57–103
3 Caro, L. H. P., Tettelin, H., Vossen, J. H., Ram, A. F. J., van den Ende, H. and Klis, F. M. (1997) Yeast **13**, 1477–1489
4 Takos, A. M., Dry, I. B. and Soole, K. L. (1997) FEBS Lett. **405**, 1–4
5 McConville, M. J. and Ferguson, M. A. J. (1993) Biochem. J. **294**, 305–324
6 Englund, P. T. (1993) Annu. Rev. Biochem. **62**, 121–138
7 Stevens, V. L. (1995) Biochem. J. **310**, 361–370
8 Udenfriend, S. and Kodukula, K. (1995) Annu. Rev. Biochem. **64**, 563–591
9 Masterson, W. J., Doering, T. L., Hart, G. W. and Englund, P. T. (1989) Cell **56**, 793–800
10 Kinoshita, T., Ohishi, K. and Takeda, J. (1997) J. Biochem. (Tokyo) **122**, 251–257
11 Milne, K. G., Field, R. A., Masterson, W. J., Cottaz, S., Brimacombe, J. S. and Ferguson, M. A. J. (1994) J. Biol. Chem. **269**, 16403–16408
12 Sharma, D. K., Smith, T. K., Crossman, A., Brimacombe, J. S. and Ferguson, M. A. (1997) Biochem. J. **328**, 171–177
13 Smith, T. K., Milne, F. C., Sharma, D. K., Crossman, A., Brimacombe, J. S. and Ferguson, M. A. (1997) Biochem. J. **326**, 393–400
14 Stevens, V. L. (1993) J. Biol. Chem. **268**, 9718–9724
15 Nakamura, N., Inoue, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V. L. and Kinoshita, T. (1997) J. Biol. Chem. **272**, 15834–15840
16 Hollander, N., Selvaraj, P. and Springer, T. A. (1988) J. Immunol. **141**, 4283–4290
17 Thomas, B. J. and Rothstein, R. (1989) Cell **56**, 619–630
18 Watanabe, R., Kinoshita, T., Masaki, R., Yamamoto, A., Takeda, J. and Inoue, N. (1996) J. Biol. Chem. **271**, 26868–26875
19 Hirose, S., Ravi, L., Hazra, S. V. and Medof, M. E. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3762–3766
20 Watanabe, R., Inoue, N., Westfall, B., Taron, C. H., Orlean, P., Takeda, J. and Kinoshita, T. (1998) EMBO J. **17**, 877–885
21 Stevens, V. L. and Raetz, C. R. (1991) J. Biol. Chem. **266**, 10039–10042
22 Boguski, M. S., Lowe, T. M. and Tolstoshev, C. M. (1993) Nat. Genet. **4**, 332–333
23 Ohishi, K., Kurimoto, Y., Inoue, N., Endo, Y., Takeda, J. and Kinoshita, T. (1996) Genomics **34**, 340–346
24 Kaiser, C., Michaelis, S. and Mitchell, A. (1994) Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25 Berben, G., Dumont, J., Gilliquet, V., Bolle, P. A. and Hilger, F. (1991) Yeast **7**, 475–477
26 Gietz, D., St. Jean, A., Woods, R. A. and Schiestl, R. H. (1992) Nucleic Acids Res. **20**, 1425
27 Gietz, R. D. and Sugino, A. (1988) Gene **74**, 527–534
28 Fenton, W. A. and Horwich, A. L. (1997) Protein Sci. **6**, 743–760
29 Riesenfeld, J., Hook, M. and Lindahl, U. (1980) J. Biol. Chem. **255**, 922–928
30 Weidanz, J. A., Campbell, P., Moore, D., DeLucas, L. J., Roden, L., Thompson, J. N. and Vezza, A. C. (1996) Br. J. Haematol. **95**, 645–653
31 Hyland, S. A., Eveland, S. S. and Anderson, M. S. (1997) J. Bacteriol. **179**, 2029–2037
32 Orlean, P., Albright, C. and Robbins, P. W. (1988) J. Biol. Chem. **263**, 17499–17507
33 Orlean, P. (1990) Mol. Cell. Biol. **10**, 5796–5805
34 Stevens, V. L., Zhang, H. and Harreman, M. (1996) Biochem. J. **313**, 253–258
35 Smith, T. K., Cottaz, S., Brimacombe, J. S. and Ferguson, M. A. (1996) J. Biol. Chem. **271**, 6476–6482
36 Vidugiriene, J. and Menon, A. K. (1993) J. Cell Biol. **121**, 987–996
37 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
38 Brandsch, R., Bichler, V., Mauch, L. and Decker, K. (1993) J. Biol. Chem. **268**, 12724–12729
39 Keresztessy, Z., Hughes, J., Kiss, L. and Hughes, M. A. (1996) Biochem. J. **314**, 41–47
40 Griepenburg, U., Blasczyk, K., Kappl, R., Huttermann, J. and Auling, G. (1998) Biochemistry **37**, 7992–7996
41 Riordan, J. F. and Vallee, B. L. (1988) Methods Enzymol. **158**, 21–32
42 Anhinsen, C. B., Edsall, J. T., Richards, F. M. and Eisenberg, D. S. (1991) in Advances in Protein Chemistry, vol. 42 (Anfinsen, C. B., Edsall, J. T., Richards, F. M. and Eisenberg, D. S., eds.), pp. 1–76, Academic Press, San Diego

Received 18 November 1998/4 January 1999; accepted 26 January 1999

© 1999 Biochemical Society