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Both Mammalian PIG-M and PIG-X are Required for Growth of GPI14-Disrupted Yeast

Youn Uck Kim[1,*], Hisashi Ashida[2], Kenichiro Mori[1], Yusuke Maeda[2], Yeongjin Hong[3] and Taroh Kinoshita[2]

[1]Department of Biological Sciences, Sun Moon University, A-San City, Korea; [2]Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; and [3]Genomic Research Center for Enteropathogenic Bacteria and Department of Microbiology, Chonnam National University Medical School, Gwangju, Korea

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GPI mannosyltransferase I (GPI-MT-I) transfers the first mannose to a GPI-anchor precursor, glucosamine-(acyl)phosphatidylinositol [GlcN-(acyl)PI]. Mammalian GPI-MT-I consists of two components, PIG-M and PIG-X, which are homologous to Gpi14p and Pbn1p in *Saccharomyces cerevisiae*, respectively. In the present study, we disrupted yeast *GPI14* and analysed the phenotype of *gpi14* yeast. The *gpi14* haploid cells were inviable and accumulated GlcN-(acyl)PI. We cloned PIG-M homologues from human, *Plasmodium falciparum* (*Pf*PIG-M) and *Trypanosoma brucei* (*Tb*GPI14), and tested whether they could complement *gpi14*-disrupted yeast. None of them restored GPI-MT-I activity and cell growth in *gpi14*-disrupted yeast. However, *gpi14*-disrupted yeast cells with human PIG-M, but not with *Pf*PIG-M or *Tb*GPI14, grew slowly but significantly when they were supplemented with rat PIG-X. This suggests that the association of PIG-X and PIG-M for GPI-MT-I activity is not interchangeable between mammals and the other lower eukaryotes.

Key words: GPI14, human PIG-M, mannose, rat PIG-X, Saccharomyces cerevisiae.

Abbreviations: GPI, Glycosylphosphatidylinositol; GPI-MT-I, GPI-mannosyltransferase I; *Pf* PIG-M, *Plasmodium falciparum* PIG-M; *Tb*GPI14, *Trypanosoma brucei* GPI14; GlcN-(acyl)PI, glucosamine-(acyl)phosphatidylinositol.

Many eukaryotic proteins are expressed on the cell surface *via* anchoring to glycosylphosphatidylinositol (GPI). Thus, GPI biosynthesis and anchoring are essential for expression of those proteins on the cell surface. GPI possesses a core structure consisting of phosphatidylinositol (PI), glucosamine (GlcN), three mannoses (Man) and ethanolamine phosphate. GPI biosynthesis is initiated by the transfer of *N*-acetylglucosamine (GlcNAc) to PI, using UDP-GlcNAc, to generate GlcNAc-PI on the cytosolic surface of the endoplasmic reticulum (ER) (*1*, *2*). The second step is de-*N*-acetylation of GlcNAc-PI to form GlcN-PI. In most eukaryotes, after acylation of inositol in GlcN-PI, the first Man is attached to GlcN-(acyl)PI from dolichol-phosphate-mannose (DPM) by GPI-mannosyltransferase I (GPI-MT-I) in the luminal side of the ER. Following this reaction, two more Man and three ethanolamines are sequentially added to the Man-GlcN-(acyl)PI (*3*). Finally, an amide group of the ethanolamine on the third Man is linked to the C-terminus of a precursor protein (*1*, *4*). An incomplete GPI synthesis affects various functions of organisms. Mice with disturbed GPI biosynthesis are embryonic lethal, indicating the importance of GPI-anchored proteins in embryogenesis (*5*). In haematopoietic stem cells, an acquired deficiency in GPI biosynthesis causes paroxysmal nocturnal haemoglobinuria (*6*). GPI biosynthesis and anchoring are also essential for *Saccharomyces cerevisiae*, because many cell wall proteins are expressed and secreted as GPI-anchored forms. *Trypanosoma brucei* causing sleeping sickness is coated by GPI-anchored variant surface glycoproteins. The blood-stage *T. brucei* is inviable with the deficiency of GPI biosynthesis and anchoring (*7–9*). Furthermore, in the *Plasmodium falciparum* causing malaria, GPI itself has been identified as toxins affecting host-cell immune responses (*10*), and malaria toxin induces the expression of proinflammatory cytokines such as TNF-α and IL-1 from macrophages (*11–13*).

Most eukaryotic organisms show a similar assembling order in GPI synthesis pathway (*1–3*), nevertheless, some steps of GPI biosynthesis are obviously different among species. For example, in *T. brucei*, the first mannosylation by GPI-MT-I occurs before inositol acylation unlike to the others. Thus, clarification of GPI biosynthetic pathways is important for developing an anti-parasite drug that could block a GPI synthesizing enzyme in a species-specific way (*14*, *15*).

Mammalian GPI-MT-I is an enzyme complex consisting of at least two components, PIG-M and PIG-X. PIG-M is a putative catalytic component, since it carries DXD motif essential in various Golgi-resident glycosyltransferases (*16*). Previously, the protein sequences of PIG-M homologues from *S. cerevisiae* (Gpi14p), *Caenorhabditis elegans* (*Ce*PIG-M) and *T. brucei* (*Tb*GPI14) have been analysed (*16*), and were also found to possess DXD motif, however, they showed a difference in ER retention signal: mammalian PIG-M and *Tb*GPI14 had no typical

*To whom correspondence should be addressed. Tel: +82-41-530-2275, E-mail: kimyu@sunmoon.ac.kr

ER retention signal, whereas Gpi14p and *Ce*PIG-M had KKXX and KXKXX sequences, respectively, at the C-terminus. Partial inactivation of GPI14 in yeast caused defects in the cell wall structure, and resulted in enhanced protein production due to the cell wall integrity-signalling pathway (*17*).

PIG-X gene encoding an ER-resident type-I transmembrane protein was identified by expression cloning using a novel GPI-defective mutant CHO cell line (*18*). PIG-X mutant CHO2.46 cells showed no GPI-MT-I activity. PIG-M was hardly expressed in CHO2.46 cells whereas it is expressed at an about 10-fold higher level in the presence of PIG-X, indicating that PIG-X plays a key role as a PIG-M stabilizer. *S. cerevisiae* Pbn1p, the sequence homologue of mammalian PIG-X, was first identified as a gene required for correct processing of protease B (PrB) in the ER, and later proposed as a chaperone-like protein for production of Gas1p and Pho8p as well as PrB (*19*, *20*). Although PBN1 alone did not complement PIG-X mutation in CHO2.46 cells, co-transfection of both PBN1 and GPI14 complement it. Similarly, GPI14 alone did not complement PIG-M mutation in Ramos517 cells, whereas co-transfection of both PBN1 and GPI14 did (*18*).

In the present study, to investigate GPI-MT-I in yeast, we have disrupted *GPI14* and biochemically analysed *gpi14* strain. Furthermore, we have cloned PIG-M homologues from human, *T. brucei* and *P. falciparum* and tested whether they could complement *gpi14*-disrupted yeast.

MATERIALS AND METHODS

Media, Strains and Transfection—The diploid strain *S. cerevisiae* W303 (*MAT***a**/*MAT*α, *ade2-1 can1-100 ura3-1 leu2-3*, *112 trp1-1 his3-11*) was obtained from the American Type Culture Collection. Media used for growing yeast strains were as follows: YPD (1% yeast extract, 2% peptone, 2% Glc), YPGal (1% yeast extract, 2% peptone, 2% Gal), and synthetic-defined medium consisting of 0.67% yeast nitrogen base (YNB, BIO 101 System) and 2% Glc or Gal with appropriate amino acid supplements (50 μg/ml). Transfection of *S. cerevisiae* was carried out by lithium acetate method.

Conditional Expression and Knock-out—pGAL (pGAL1, pGALL and pGALS) vector carrying Gal-inducible promoters and 2 micron was used for conditional expression in *S. cerevisiae* (*21*). Cells containing a galactose-regulated gene are not expected to grow in glucose medium, since the gene expression should be off. Growth could be dependent on residual gene expression. For disrupting *GPI14* gene, 959 bp in the coding region of *GPI14* (between EcoRV site at 100 nt downstream from the start codon and EcoRI site at 140 nt upstream from the stop codon) was replaced with *LEU2* gene.

Construction of PIG-Ms and PIG-X Expression Vectors—A candidate of PIG-M homologue in the genome of *P. falciparum*, ORF name *Pf*PIG-M or accession number AB281496, was cloned by following procedure. The cDNA was amplified for three separated fragments by PCR using *P. falciparum* cDNA library constructed in a p426 Met25 yeast vector (from Dr Ralph T. Schwarz. in Philipps-Universität, Germany) as a template. Three fragments were amplified using following primers: MET25-5, 5′-TAA AGC GTC TGT TAG AAA GG-3′ and Mal-3, 5′-GAT AAC ATC TGC ATT ACC TCG AGG GGA TAT-3′ for 1–450 nt; Mal-4, 5′-ATC ACC ATA TGA AAG ATC TAG ATA TAG ATA-3′ and Mal-2, 5′-CTT CTC AAT ACT CGA GAT CAT AAG GTA AAA-3′ for 250–1150 nt; Mal-1, 5′-TAT ATC AGA TCT AGA TAT GAA TTT TTG TAT-3′ and CYC-1, 5′-GAG CGT CCC AAA ACC TTC TCA AGC AAG-3′ for 850 nt to the stop codon. Amplified three cDNA fragments were digested with XbaI, ClaI and EcoRI, and ligated into pBluescript KS plasmid. FLAG sequence were inserted into PstI and SalI sites using the oligonucleotide 5′- CTG CAG ACG CGT GAC TAC AAG GAC GAC GAT GAC AAG GTC GAC-3′ for C-terminal tag. For inserting the cloned cDNA to pGAL vector, coding regions were amplified using primers that carried EcoRI site and start codon at 5′ terminus, and stop codon and SalI site at 3′ terminus, digested with these enzymes and ligated. The yeast *GPI14* and *T. brucei TbGPI14* were obtained from their genomic DNA by PCR as described (*14*, *16*). For construction of PIG-X expression vector, rat PIG-X cDNA clone 63B(CTG→ATG) was used as a template, because the wild-type clone 63B has an alternative initiation codon, CTG (*18*).

Western Blot Analysis—The cell pellet from 0.5-ml culture was crushed with glass beads in the presence of 100 μl of SDS–PAGE sample buffer and heated at 95°C for 5 min. After centrifugation, 10 μl of supernatant was subjected to 10–20% gradient SDS–PAGE gel and electroblotted onto a polyvinylidene difluoride membrane. The blot was treated with the primary antibody, anti-FLAG BioM2 (Sigma–Aldrich), and then with horseradish peroxidase-conjugated protein G (Bio-Rad). Detection was carried out by using western lightning chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Boston, MA).

Tetrad Analysis of Transfected Saccharomyces cerevisiae—Tetrad analysis was carried out by the methods described previously (*22*). Detection of rat PIG-X and human PIG-M cDNAs in the 4 asci was performed by PCR using following primers: forward 5′-GCC GCA GAC ATC AGC GAC GCC-3′ and reverse 5′-GAC CAC AAT GAG GGT GTC TCC-3′ for rat PIG-X (expected product, 420 bp); forward 5′-CTA GCC AGA GTC GCC CTG-3′ and reverse 5′-AGT GTA CCG GAA TTG ACG-3′ for human PIG-M, (545 bp); forward 5′-ATT GAA TCC GAT GGT TAT-3′ and reverse 5′- CAA GCA GCA ATT GAG GCA-3′ for GPI14, (455 bp), respectively. For confirming *Leu-2* gene, the following primers were used: forward 5′-TAG GCT TTC TTG ATC AAT-3′ and reverse 5′-CTG TCG CCG AAG AAG TTA-3′ (870 bp).

Metabolic and In vitro Labelling—Yeast cells were grown in inositol-free YNB-Glc medium for metabolic labelling. Cells in 250 μl medium were then pre-incubated in inositol-free medium for 20 min and radio-labelled with the addition of [^{3}H]inositol (5 μCi) followed by 4-fold dilution with the same medium. To stop the labelling, cells were placed on ice and NaN_3 and NaF (10 mM final) were added. After washing with 1-M sorbitol solution, same amount of cells were subjected

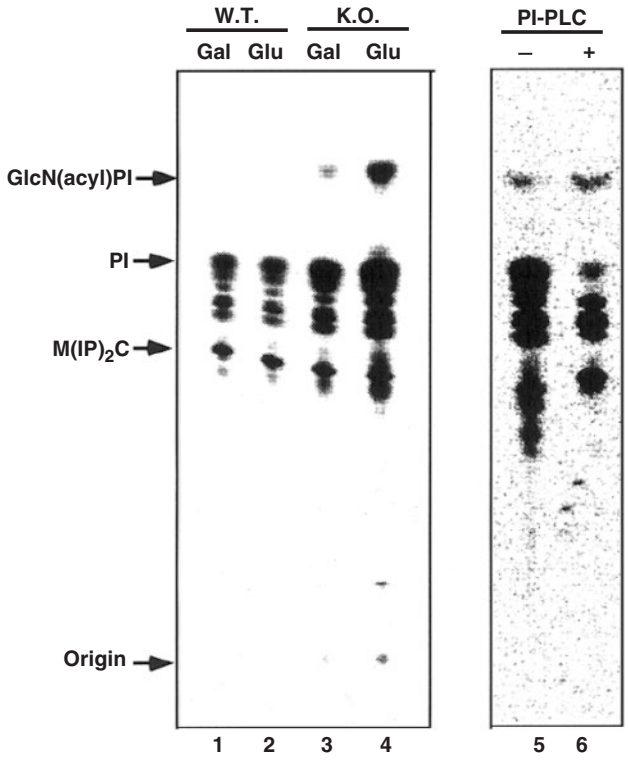

Fig. 1. **Metabolic labelling of GPI intermediates with radiolabelled inositol.** The haploid yeast cells transfected with pGAL(Ura)-*GPI14* were labelled with [^{3}H]inositol in inositol-free YNBA.A. medium supplemented with Gal or Glc, and radiolabelled lipid fractions were extracted for TLC analysis (lanes 1–4). The same lipids from lane 4 were treated by PI-PLC (lanes 5 versus 6). Lanes 1 and 2, wild-type haploid cells transfected with pGAL(Ura)-*GPI14*-FLAG; lanes 3 and 4, *gpi14*-disrupted haploid cells transfected with pGAL(Ura)-*GPI14*-FLAG.

to lipid extraction and analysed by TLC. For labelling of GPI intermediates *in vitro*, the microsomes obtained from cells (2×10^7) were incubated in 50 µl buffer, containing 2 µCi of GDP-[^{3}H]Man (American Radiolabeled Chemicals), 50 mM HEPES–NaOH (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM 5′AMP, 1 µg/ml tunicamycin, 10 µM palmitoyl-CoA, 2 µg/ml leupeptin and 0.1 mM TLCK, at 37°C. Some reactions also contained 0.1 µg of GlcN-PI(C8) (a gift from Dr M. A. Lehrman, University of Texas Southwestern) which was added as a solution in 0.03% Triton X-100. After 1 h incubation, lipids were extracted and analysed by TLC. Radioactivity was detected by two-dimensional radio scanning (LB 2842; Berthold AG, Regensdorf, Switzerland).

Enzyme Treatments of Labelled Lipids—PI specific phospholipase C (PI-PLC), GPI specific phospholipase D (GPI-PLD), and jack bean α-mannosidase (JBAM) treatments were carried out according to the procedures described previously (*23–25*). After incubation with enzymes for 12–16 h at 37°C, lipids were extracted with 1-butanol and analysed as described earlier.

RESULTS

The gpi14-deficient Yeast Cells Accumulate GlcN-(acyl)PI—We first determined whether *GPI14* is an essential gene for growth. We generated diploid heterozygous *GPI14/gpi14::LEU2* strain and assessed it by tetrad analysis. The only two haploid cells grew from dissected asci, indicating that *GPI14* is essential for growth (data not shown). This is consistent with previous reports on comprehensive gene disruption (*26*, *27*) and on specific study on *GPI14* (*17*).

We next analysed whether GlcN-(acyl)PI, a substrate of GPI-MT-I, was accumulated in *gpi14*-disrupted haploid cells. For this, we established wild-type and *gpi14*-disrupted haploid cells transfected with pGAL(Ura)-*GPI14*. Cells were metabolically labelled for 20 min with [^{3}H]inositol in inositol-free YNB-Gal or YNB-Glc media. The expression of *GPI14* gene from pGAL(Ura)-*GPI14* can be turned on and off in Gal and Glc containing media, respectively. As expected, GlcN-(acyl)PI in the wild-type haploid cells was not detected (Fig. 1, lanes 1 and 2). In contrast, GlcN-(acyl)PI in *gpi14*-disrupted haploid cells was slightly accumulated even in Gal-containing medium (lane 3) and was much highly accumulated in Glc-containing medium (lane 4). The identity of the GlcN-(acyl)PI was confirmed by the fact that it was resistant to PI-PLC (lanes 5 and 6). These results indicate that the Gpi14p deficiency resulted in the GlcN-(acyl)PI accumulation and that the Gpi14p is a GPI-MT-I component in yeast.

PIG-M Homologues did not Complement gpi14-deficient Yeast Cells—Previously we identified human PIG-M and its homologue *Tb*GPI14 in *T. brucei*. We further searched a genomic database of *P. falciparum* to identify PIG-M homologue. One candidate (ORF *Pf*PIG-M or accession no. AB281496) was found, which shows 35% amino acid identity to human PIG-M and 36% to yeast Gpi14p. To elucidate whether these PIG-M homologues could restore the function of *gpi14*-deficient yeast, each PIG-M was tagged with FLAG at the C-terminus and transfected to the wild-type haploid cell. All FLAG-tagged proteins were well expressed in the wild-type yeast (Fig. 2). Next, we transfected *GPI14/gpi14::LEU2* diploid cells with the same vectors and dissected the asci. However, none of the cells could form four colonies from an ascus except for *GPI14* transfectant (data not shown), indicating that these PIG-M homologues could not restore the lethality caused by the *gpi14* deficiency in yeast.

Next, we analysed the synthesis of Man-GlcN-(acyl)PI in the *gpi14*-disrupted yeast cells, which have been transfected with PIG-M homologues. For this experiment, *gpi14::LEU2* haploid cells harbouring pGAL(Ura)-*GPI14* were transfected with PIG-M homologues using pGAL(Trp) vector (Fig. 3A). The microsomes were prepared from these transfectants cultured in YNBGal-Leu-Ura-Trp media, and were incubated with GDP-[^{3}H]Man for analysis of mannolipids formation. Several spots were detected on TLC (Fig. 3B). Among them, two spots sensitive to both GPI-PLD and JBAM corresponded to Man-GlcN-(acyl)PI and Man_2-GlcN-(acyl)PI [indicated as M1(endo) and M2(endo), respectively] (Fig. 3C, lanes 1, 2, 5 and 6). The amounts of M1(endo) and M2(endo)

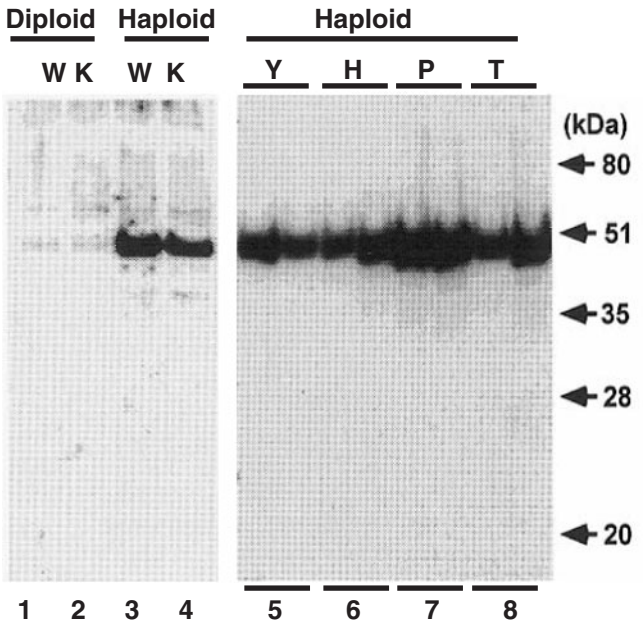

Fig. 2. **Expressions of PIG-M homologues in *S. cerevisiae*.** pGAL(Ura) expression vectors carrying FLAG-tagged GPI14 (Y), hPIG-M (H), *Pf*PIG-M (P) or *Tb*GPI14 (T) were transfected to wild-type haploid cells. Proteins were analysed by western blotting using anti-FLAG antibody. We did not detect any specific band in non-transfected wild (W) or *GPI14*-disrupted (K) diploid cells (lanes 1 and 2). Two kinds (W and K) of haploid cell derived from diploid cells that *gpi14* disrupted and rescued by pGAL(Ura)–*GPI14* were shown in lanes 3 and 4, respectively. Lane 5, GPI14-FLAG; lane 6, hPIG-M-FLAG; lane 7, *Pf*PIG-M-FLAG; lane 8, *Tb*GPI14-FLAG.

were increased only in the microsomes carrying both pGAL(Ura)-*GPI14* and pGAL(Trp)-*GPI14* (Fig. 3B, lanes 1 versus 2), but not in others (lanes 1 versus 3–5). We next added GlcN-PI(C8), a synthetic acceptor substrate for GPI-MT-I after inositol acylation, to the reaction mixtures, because the endogenous substrate might be limiting (*28*). New spots derived from GlcN-PI(C8) appeared (Fig. 3B, lanes 6–10) and they were identified to be Man-GlcN-(acyl)PI(C8) and Man_2-GlcN-(acyl)PI(C8) [indicated as M1(C8) and M2(C8), respectively] by GPI-PLD and JBAM treatments (Fig. 3C, lanes 3–6). The amounts of M1(C8) and M2(C8) were increased only in the microsomes carrying both pGAL(Ura)-*GPI14* and pGAL(Trp)-*GPI14* (Fig. 3B, lanes 6 versus 7), but not in others (lanes 6 versus 8–10). The asterisked mannolipid with similar mobility to M2(C8) seen in lanes 1–5 (Fig. 3B) is not characterized. These results indicate that human PIG-M, *Tb*GPI14 and *Pf*PIG-M did not exhibit GPI-MT-I activity in yeast although the proteins were highly expressed.

Co-transfection of Mammalian PIG-M and PIG-X Partially Restored gpi14-deficient Yeast Cells—GPI-MT-I in mammalian cells consists of at least two components PIG-M and PIG-X and the stable expression of PIG-M is dependent upon PIG-X (*18*). Since the growth of *gpi14*-deficient yeast was not restored by human PIG-M, we speculated that PIG-X is also required for the function of human PIG-M in yeast. To test

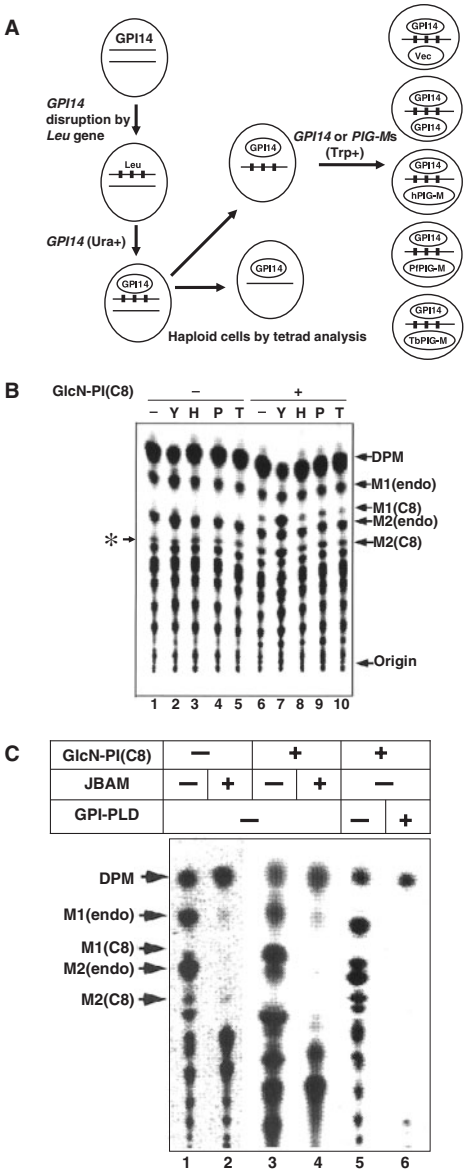

Fig. 3. **PIG-M homologues did not complement *gpi14*-deficient yeast cells.** (**A**) Schematic procedure for preparing *gpi14*-disrupted haploid cells carrying *GPI14* and one of *PIG-M* homologues. (**B**) *In vitro* mannosylation in *gpi14*-disrupted haploid cells carrying both GPI14 and PIG-M homologue. The microsome fractions were mixed with GDP-[^{3}H]Man in the absence (lanes 1–5) or presence (lanes 6–10) of GlcN-PI(C8). After incubation for 1 h, labelled mannolipids were extracted and analysed by TLC. Lanes 1 and 6, pGAL(Trp) empty vector; lanes 2 and 7, pGAL(Trp)-*GPI14*-FLAG; lanes 3 and 8, pGAL(Trp)-h*PIG-M*-FLAG; lanes 4 and 9, pGAL(Trp)-*PfPIG-M*-FLAG; lanes 5 and 10, pGAL(Trp)-*TbGPI14*-FLAG. M1(endo) and M1(C8) represent Man-GlcN-(acyl)PI from endogenous source and GlcN-PI(C8), respectively. M2(endo) and M2(C8) represent Man-GlcN-(acyl)PI from endogenous source and GlcN-PI(C8), respectively. Identity of the mannolipid with mobility similar to M2(C8) seen in lanes 1–5 (∗) is unclear. (**C**) Characterization of mannolipids by enzyme treatments. The radio-labelled mannolipids identical to lanes 2 and 7 in the previous panel were treated with JBAM and GPI-PLD. − or + represents absence or presence of each enzyme. Different profiles of mannolipids in lanes 3 and 5 were due to effects of different buffers.

this hypothesis, we transfected *GPI14*/*gpi14::LEU2* diploid cells with both pGAL(Ura)-*PIG-M* and pGAL(His)-*PIG-X*, and then four haploid cells were obtained by tetrad analysis method (Fig. 4A). Two large and two tiny haploid colonies were formed on YPGal plate (Fig. 4B). Two tiny colonies, but not two large ones, grew significantly in YPGal-Leu-Ura-His plate, confirming that they had *gpi14*-disrupted background (Fig. 4C). These two tiny colonies did not grow on YPD plate, further confirming that growth on YPGal plate was in fact dependent upon PIG-M and PIG-X expression plasmids (Fig. 4C). The genotypes were further confirmed by PCR: tiny colony had *PIG-X*, *PIG-M* and *LEU2* genes, but not *GPI14*; large colony had *PIG-X*, *PIG-M* and *GPI14* genes (Fig. 4D). These results clearly indicate that PIG-X is required for the function of PIG-M in yeast. Next, we tested whether co-transfection with *Pf*PIG-M and rat PIG-X restored the growth of *gpi14*-disrupted cells. We performed tetrad analysis in a similar way. However, we observed only two colonies in YPGal plate, indicating that mammalian PIG-X did not support the function of *Pf*PIG-M (data not shown).

DISCUSSION

Three Man residues in the GPI core are linked through α-1,4, α-1,6 and α-1,2 bonds. GPI-MT-I, -II and -III involved in formation of these linkages are named Gpi14p (PIG-M in mammals), Gpi18p (PIG-V) and Gpi10p (PIG-B), respectively (*16*, *29*, *30*). As in Gpi18 and Gpi10, we experimentally showed that Gpi14 is essential for yeast cell viability. Lethality of *gpi14* mutant must be due to the defect of GPI-anchor biosynthesis, since *gpi14* accumulated GlcN-(acyl)PI, the substrate for GPI-MT-I (Fig. 1). The fourth α-1,2 linked Man on GPI anchor is transferred by Smp3p (SMP3 in mammals), which is also essential for yeast cell viability but not for mammals (*31*). These mannosyltransferases have similar membrane organization, namely they are multiple transmembrane proteins, and they form a superfamily in glycosyltransferases (*32*). There are, however, almost no sequence similarity among them, except for Gpi10p and Smp3p.

Since the sugar moiety of GPI is well conserved among all eukaryotes, many homologous mannosyltransferases are interchangeable between yeast and mammals. Human PIG-V, PIG-B and SMP3 partially complemented the growth defect of *gpi18*, *gpi10* and *smp3* yeast mutants, respectively (*29*, *14*, *33*). In contrast to GPI-MT-II, -III and -IV, GPI-MT-I is rather different. Unexpectedly, *gpi14* mutant was not complemented by human PIG-M, and also not by *Tb*GPI14 and *Pf*PIG-M even under over-expression conditions (Fig. 3). Since we previously identified that PIG-X is required for GPI-MT-I activity in mammalian cells (*18*), we tried to complement *gpi14* mutant by co-transfection of mammalian PIG-M and PIG-X. As expected, co-transfection of two molecules partially rescued the growth defect of *gpi14* mutant (Fig. 4). The partial rescue may be due to heterologous combination of human PIG-M and rat PIG-X and/or inefficient action of mammalian GPI-MT-I in yeast cells. These results suggest that Pbn1p, the yeast homologue of

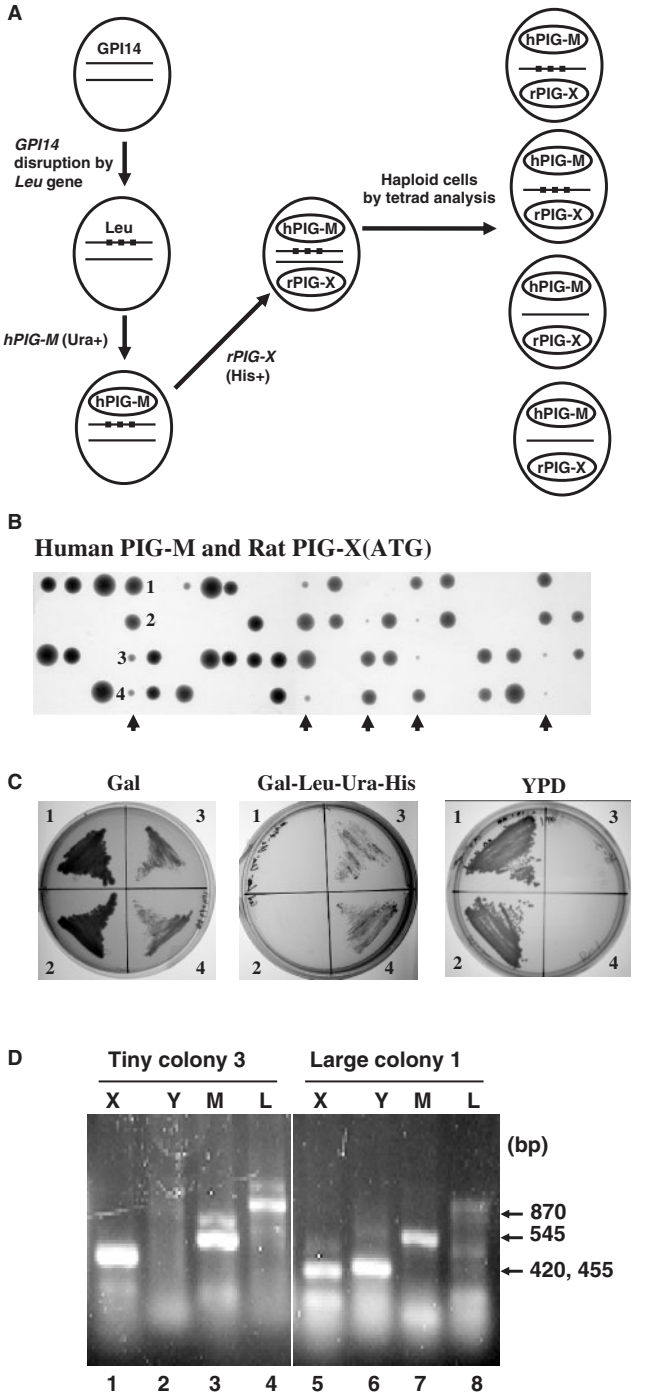

Fig. 4. **Co-transfection with both mammalian PIG-M and PIG-X partially restored the growth of the *gpi14*-disrupted cells.** (**A**) Schematic procedure for preparing *gpi14*-disrupted haploid cells carrying both mammalian *PIG-M* and *PIG-X*. (**B**) The tetrad analysis of *GPI14*/*gpi14::LEU2* diploid cells carrying human PIG-M and rat PIG-X. Arrowheads indicate sets of spores formed two large and two tiny colonies. (**C**) Growth of four colonies from an ascus (numbered in panel B). Left, YNBGal plate; centre, YNBGal plate without Leu, Ura and His; right, YPD plate. (**D**) Genotype analysis of colony numbers 3 (tiny colony) and 1 (large colony) in panel B. PCR was done using primer pairs for rat *PIG-X* (X), *GPI14* (Y), human *PIG-M* (M) and *LEU2* (L) genes. The expected size of PCR products were indicated at right side.

mammalian PIG-X, does not form the enzyme complex with PIG-M from other organisms or does not stabilize them. It was reported that a lack of Pbn1p affected processing of several vacuolar proteins, such as protease B (Prb1p) and alkaline phosphatase (Pho8p), which are not GPI-anchored proteins (*20*). They speculated that Pbn1p is required for proper folding and/or the stability of a subset of proteins in the ER, although it is not essential for global exit of proteins from there. Since *PRB1* and *PHO8* are non-essential genes for cell viability, one of the essential roles of *PBN1* must be the function in GPI-MT-I.

We searched Pbn1p homologue in the genomic databases of *P. falciparum* and *T. brucei*, but could not find any candidate. The substrate specificity of GPI-MT-I from *T. brucei* was reported to be different from those of mammals: the former prefers non-acylated GlcN-PI whereas the latter acylated GlcN-PI (*34*). Thus, the protozoan parasites may have different sequence in GPI-anchor biosynthesis and may have unique subcomponent of GPI-MT-I. This suggests that GPI-MT-I might be a promising target for anti-parasite drug development. Similarly, yeast GPI-MT-I also could be a target for anti-fungal drug because the combination or assembly of Gpi14p and Pbn1p is highly specific.

Recently, a mycobacterial mannosyltransferase PimE distantly related to Gpi14p/PIG-M was identified, which is involved in the biosynthesis of phosphatidylinositol mannoside (PIM) (*35*). Gpi14p/PIG-M and PimE may have a common ancestral gene and may be functionally diversified in the world of eukaryotes and prokaryotes. They may share the recognition sites for polyprenol-phosphate-mannose and PI. Further biochemical and structural studies might be important.

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