Inositol Deacylation of Glycosylphosphatidylinositol-anchored Proteins Is Mediated by Mammalian PGAP1 and Yeast Bst1p*

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The inositol moiety of mammalian glycosylphosphatidylinositol (GPI) is acylated at an early step in GPI biosynthesis. The inositol acylation is essential for the generation of mature GPI capable of attachment to proteins. However, the acyl group is usually absent from GPI-anchored proteins (GPI-APs) on the cell surface due to inositol deacylation that occurs in the endoplasmic reticulum (ER) soon after GPI-anchor attachment. Mammalian GPI inositol-deacylase has not been cloned, and the biological significance of the deacylation has been unclear. Here we report a GPI inositol-deacylase-deficient Chinese hamster ovary cell line established by taking advantage of resistance to phosphatidylinositol-specific phospholipase C and the gene responsible, which was termed PGAP1 for Post GPI Attachment to Proteins 1. PGAP1 encoded an ER-associated, 922-amino acid membrane protein bearing a lipase consensus motif. Substitution of a conserved putative catalytic serine with alanine resulted in a complete loss of function, indicating that PGAP1 is the GPI inositol-deacylase. The mutant cells showed a clear delay in the maturation of GPI-APs in the Golgi and accumulation of GPI-APs in the ER. Thus, the GPI inositol deacylation is important for efficient transport of GPI-APs from the ER to the Golgi.

Many eukaryotic cell surface proteins with various functions are anchored to the membrane via glycosylphosphatidylinositol (GPI) (1–3). GPI-anchored proteins (GPI-APs) on mammalian cells are usually sensitive to bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), leading to the release of the protein portions. Therefore, PI-PLC is often used as a tool to determine whether proteins are GPI-anchored. In contrast, precursors of the GPI-anchor present in the endoplasmic reticulum (ER) are resistant to PI-PLC due to an acyl chain linked to the 2-position of inositol (4). The inositol ring of GPI is acylated at an early step in GPI biosynthesis by the action of PGAP-W protein, an acyltransferase that adds a palmitoyl chain to the inositol of glucosaminy1-phosphatidylinositol, the second intermediate in the pathway (5). The inositol acylation is critical for the attachment of GPI to proteins, because mutant cells defective in PG-W express only very low levels of GPI-APs (5). It is very likely that the acyl group is required for a later step in the pathway when “bridging” ethanolamine phosphate, which links GPI to the protein, is added to the third mannose to generate mature GPI. Soon after the attachment of GPI to proteins, the inositol is usually deacylated in the ER and becomes sensitive to PI-PLC (6). Human erythrocytes represent an exception, in which the inositol remains acylated, and all the GPI-APs are resistant to PI-PLC (7–9). A possible reason for the lack of deacylation in human erythrocytes is that GPI-APs bearing three acyl chains are more stably associated with the membrane than those bearing two acyl chains, and thus the maintenance of GPI-APs during the long life of erythrocytes is ensured.

The enzyme involved in inositol deacylation of GPI-APs has not been characterized. Furthermore, the reason why the deacylation usually occurs is unclear. In order to identify a gene for the GPI inositol-deacylase, we isolated a mutant Chinese hamster ovary (CHO) cell line defective in inositol deacylation and cloned the gene responsible, PGAP1 (for Post GPI Attachment to Proteins 1). Rat PGAP1 is an ER membrane protein with a catalytic serine-containing motif that is conserved in a number of lipases.

MATERIALS AND METHODS

Cells and Culture—Wild-type CHO-K1, 3B2A (10), and C10 cells (see below for characterization) were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 0.5 mg/ml G418 (3B2A and C10 cells only). To establish an RD3 rescued cell line, C10 cells were electroporated with 20 μg of linearized pME-Puro-PGAP1 for the expression of PGAP1, selected in Ham’s F-12 medium with 5 μg/ml of puromycin for 1 week, and cloned by limiting dilution.

Plasmids—The plasmids pME-Py (an expression vector with a polyoma origin of replication), pME-Py-FLAG-ALDH (ALDH, microsomal aldehyde dehydrogenase) (11), and pME-Py-GST-FLAG-hPIG-M were described previously (12, 13). A plasmid pME-Puro-PGAP1 was constructed by subcloning the XhoI/XbaI fragment of pME-Py-PGAP1 into the XhoI/XbaI site of pME-Puro. To fuse a FLAG tag at the N terminus of the rat, the rDPM2 cDNA of pME-Puro-RDPM2 was replaced with a cDNA of PGAP1 whose start codon was replaced by a SalI recognition sequence. A glutathione S-transferase (GST)-tagged PGAP1 was constructed in a similar way using pME-Py-GST-RDPM2. We termed these plasmids pME-Py-FLAG-PGAP1 and pME-Py-GST-PGAP1, respectively.

To obtain the Bst1p expression vector pME-Py-BST1, we amplified BST1 with the primers 5’-GGGGCGCGCCAATGGGATATCCAGGAGATTGTGGTTGTT-3’ (forward) and 5’-TCTAGACTAATGTATTGTTTCGAAAGATGAC-3’ (reverse) from a Saccharomyces cerevisiae genomic DNA library, and we cloned it into the pGEM-T Easy vector (Promega). The BST1 was sequenced and subcloned into the NotI/XbaI site of pME-Py.

We substituted serine 174 in PGAP1 with alanine by site-directed
mutagenesis using a Quick Change Site-directed Mutagenesis Kit (Stratagene). After sequencing, the ClaI/BglII fragment of the mutated plasmid was used to replace the corresponding part of the wild-type pME-Py-PGAP1 to obtain pME-Py-PGAP1 (S174A).

To construct pME-Puro-FLAG-folate receptor 1 (FR1), we amplified the human FR1 sequence with primers 5' -CCAGAAGCTTCCGAGGTCAGGA -GAAGATGCTATCGG-3' (forward) and 5' -GGAGCTGCCTCGAGGACCAAGCCACAGCA-3' (reverse) from human cDNA library. The HindIII/NotI-cut fragment was subcloned into HindIII/NotI-cut pME-Puro-FLAG-CDS9 to replace CDS9 with FR1.

Establishment of a GPI Inositol-deacylation-defective Cell Line, C10—

3B2A cells (10^7) were treated with 0.4 mg/ml ethyl methanesulfonate for 1 day, followed by a 6-day culture without ethyl methanesulfonate. Cells were treated with 1 unit/ml PI-PLC for 15 min at 37 °C, and the PI-PLC-resistant cells were enriched by auto-MACS (Miltenyi Biotec) using biotinylated anti-CD59 (5H8) and anti-DAF (IA10) antibodies and cloned by limiting dilution.

Cloning of PGAP1 cDNA—C10 cells (1.2 x 10^8) were mixed with 360 μg each of a rat C6 glial cDNA library (10) and pcDNA-Py/Tori→ plasmids (10) in 4.8 ml of Opti-MEM I and electroporated in 12 cuvettes at 960 microfarads and 300 V using Gene Pulser (Bio-Rad). After 1 day, cells (10^6/ml) were treated with 25 mg/ml Pronase (Sigma) in Opti-MEM 1 for 2 h at 37 °C. Transfected cells were resuspended in Opti-MEM 1 at 10^7/ml 1.5 days after the Pronase treatment, treated with 1 unit/ml PI-PLC for 1 h at 37 °C, and then stained with a biotinylated anti-CD59 antibody (5H8) and phycoerythrin-conjugated streptavidin (Biomeda). Approximately 6,000 cells with low negative CD59 staining were collected using a cell sorter (FACS-Vantage, BD Biosciences). Plasmids (2 x 10^9) were recovered from these cells by Hirt's method (14). Pooled plasmids (15 μg) were transfected with 45 μg of pcDNA-Py/Tori→ plasmids into 1.8 x 10^6 C10 cells, as described above, in 9 cuvettes. After another cycle of cell sorting and plasmid recovery, the plasmids were electroporated into Esherichia coli and fractionated to 576 pools (20–30 clones/pool). One of these pools was obtained from this pool. Human PGAP1 cDNA—Human PGAP1 cDNA was amplified by PCR using primers 5' -GGAGTGCGGCCGCTAG-3' (forward) and 5' -GGCGCCGCTACATAAAAGTTGCATATAGC-ATGG-3' and reverse (5' -GGCGCCGCTACATAAAAGTTGCATATAGC-ATGG-3') primers. The sequences were determined based on the ones of FLJ12377 (DDJB/GenBank™/EMBL accession number AK022439) and IMAGE:1678930 (3') (DDJB/GenBank™/EMBL accession number AI076615), respectively.

PI-PLC Sensitivity Assay—Cells (10^6) were treated with or without 50 μl of 1 unit/ml PI-PLC in Opti-MEM I (Invitrogen) for 1–1.5 h at 37 °C. The cells were stained for CD59 and DAF and analyzed with a FACS Calibur (BD Biosciences).

Triton X-114 Partitioning of FLAG-tagged CDS9—C10 cells (2 x 10^6) were transiently transfected with 3.2 μg of pME-Py-Py-PGAP1-CDS9 (15) using DECTAMINE 2000 (Invitrogen) and harvested after 72 h. The cells were lysed in 2% Triton X-114 (Nacalai Tesque) in Opti-MEM I for 10 min at 37 °C, washed in 1% bovine serum albumin in PBS for 30 min, and analyzed by SDS-PAGE/Western blotting.

Alkaline Hydroxylamine Treatment of FLAG-FR1—3B2A and C10 cells were transfected with FLAG-FR1 expression plasmid. After 2 days, cells (10^6) were treated with or without 1 μl hydroxylamine (Sigma) in 0.1 x triethylamine, pH 11.5, overnight at 4 °C. After neutralizing, the plasma membranes were recovered by centrifugation at 100,000 x g for 1 h at 4 °C, washed, and resuspended in 100 μl of PBS by sonication, treated with or without 3 units/ml PI-PLC for 1.5 h at 37 °C. FLAG-FR1 proteins were subjected to Triton X-114 partitioning and analyzed by SDS-PAGE/Western blotting as described above.

Subcellular Localization of PGAPI—CHO cells (4 x 10^6) transfected with FLAG-PGAPI were fractionated, and the activity of plasma membrane, Golgi, and ER marker enzymes were assayed as described previously (16). FLAG-PGAPI was extracted and immuno-precipitated from each fraction using anti-FLAG beads and then analyzed by SDS-PAGE/Western blotting.

Pulse-chase Analysis of DAF—C10 and RD3 cells were incubated for 30 min at 37 °C in methionine- and cysteine-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% dialyzed fetal bovine serum, pulse-labeled with 100 μCi/ml [35S]methionine and -cysteine (Amersham Biosciences) for 10 min at 37 °C, and then chased with cold 1.5 x methionine and 1.2 x cysteine for 0–60 min at 37 °C. Cells were washed with ice-cold PBS and lysed in 0.6 ml of RIPA buffer (20 μl Tris-HCl, pH 7.4, 150 μl NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing "complete protease inhibitor mixture" (Roche Applied Science). DAF was immunoprecipitated using a monoclonal anti-DAF antibody (IA10) and protein-A-Sepharose (Amersham Biosciences). Non-cross-linked DAF was separated by SDS-PAGE under reducing conditions and analyzed using a BAS 1000 analyzer (FujiFilm).

Membrane Orientation of PGAPI Protein—CHO cells were transiently transfected with pME-Py-GST-PGAPI or pME-Py-GST-FLAG-HPIG-M and replated on gelatin-coated coverslips after 1 day of culture. After 2 days, cells were fixed with 4% paraformaldehyde in PBS for 15 min and then NH,Cl in PBS for 30 min. Nonspecific binding sites were blocked with 1% bovine serum albumin in PBS for 30 min. For complete permeabilization, 0.1% Triton X-100 was added to the blocking solution and incubated for 30 min at room temperature. For selective permeabilization of the plasma membrane, cells were incubated in 5 μg/ml digitonin (Wako) for 30 min. The cells were then incubated with a rabbit anti-BIP antibody (ABR) or goat anti-GST antibody (Amersham Biosciences) followed by rhodamine-conjugated donkey anti-rabbit IgG (Chemicon) or Alexa 488-conjugated donkey anti-goat IgG (Molecular Probes) antibodies. The cells were observed under a fluorescent microscope (BX-50, Olympus) equipped with a CCD camera (VB-8010, Keyence).

RESULTS

Establishment of a PI-PLC-resistant Cell Line, C10—To identify a GPI inositol-deacylase, we established a cell line whose surface GPI-APs were not released by PI-PLC, because GPI-APs on the cell surface would become resistant to PI-PLC if the cells lacked a GPI inositol-deacylase. We mutagenized 3B2A cells, CHO-K1-derived cells expressing CD59 and DAF (10), treated them with PI-PLC, and enriched the cells whose CD59 and DAF were resistant to PI-PLC by repeated Magnetic Cell Sorting. We obtained eight clones affected PI-PLC (lanes 6 and 8) and termed C10, in the following experiments.

We confirmed the PI-PLC resistance of C10 cells by FACS. The intensities of CD59 and DAF on C10 mutant cells were similar to those of wild-type 3B2A cells and were not affected by PI-PLC treatment (Fig. 1A, a and b), whereas the intensities on 3B2A cells were decreased by 90–95% (c and d).

We further confirmed the PI-PLC resistance of the FLAG-tagged CDS9 (FLAG-CDS9) extracted from C10 cells by Triton X-114 partitioning (Fig. 1B). Most of the mature FLAG-CDS9 from 3B2A cells was partitioned into the detergent phase without PI-PLC treatment (lanes 3 and 4), whereas the majority of the mature FLAG-CDS9 was partitioned into the aqueous phase after treatment with PI-PLC (lanes 1 and 2). In contrast, a major part of the FLAG-CDS9 proteins from C10 cells was partitioned into the detergent phase even after PI-PLC treatment (lanes 6 and 8). The smaller amount of mature FLAG-CDS9 in C10 cells suggested that the maturation of FLAG-CDS9 was affected in these mutant cells (see below for further results).

We found two bands of smaller molecular size (arrows a and b). Band a was detected in both cell lines and was hydrophilic (lanes 3 and 7). It may be FLAG-CDS9 that failed to become GPI-anchored due to the overexpression. Band b was hydrophobic, PI-PLC resistant, and only seen in C10 mutant cells (lanes 6 and 8). This would be an immature GPI-anchored FLAG-CDS9 bearing acylated inositol.

To obtain evidence that the PI-PLC resistance of GPI-APs on C10 mutant cells is indeed due to the inositol acylation, we eliminated the acyl chain by treating C10 cells with alkaline hydroxylamine (17) and then tested whether GPI-APs became PI-PLC-sensitive. We transfected C10 and 3B2A cells with FLAG-tagged FR1, GPI-AP. Some of the FLAG-FR1 was lost after alkaline hydroxylamine treatment, most likely because FR1 with GPI-anchor having alkylacylglycerol was released and because FR1 with GPI-anchor having alkyacylglycerol re-
mained membrane-bound. We analyzed PI-PLC sensitivity of the remaining FLAG-FR1 by Triton X-114 partitioning. Although FLAG-FR1 on alkali buffer-treated C10 cells was resistant to PI-PLC (Fig. 2, lanes 5–8), the majority of FLAG-FR1 became PI-PLC-sensitive after alkaline hydroxylamine treatment (lanes 1–4). FLAG-FR1 on 3B2A was sensitive to PI-PLC without alkaline hydroxylamine treatment as expected (lanes 9–12). These results support that the inositol acylation of GPI-APs on C10 mutant cells was the basis for PI-PLC resistance.

Expression Cloning and Characterization of PGAP1 cDNA—To obtain cDNAs that rescued the defect in C10 mutant cells, we transfected C10 mutant cells with a rat cDNA library, collected PI-PLC-sensitive cells with a cell sorter, and recovered the plasmids. This plasmid pool was used for the second round of enrichment. We finally obtained three clones with the same insert size and restriction profile, suggesting that these plasmids were derived from the same clone. We termed the gene PGAP1. We established a C10-derived cell line, termed RD3, which stably expressed PGAP1. The PI-PLC sensitivity of CD59 on RD3 cells was slightly higher than that of 3B2A wild-type cells (Fig. 3A, b and c), indicating that PGAP1 rescued the defect in C10 mutant cells. The hypersensitivity of RD3 to PI-PLC may be due to the overexpression of PGAP1. The PGAP1 cDNA restored PI-PLC sensitivity on seven other mutant clones, indicating that all eight mutant cells were defective in PGAP1 gene (data not shown).

The PGAP1 cDNA consisted of 3260 bp, and the longest open reading frame, spanning nucleotides 21–2789, encoded 922 amino acid residues (DBJ/GenBankTM/EMBL accession number AB116149; Fig. 3B). The nucleotide sequence around the start codon agreed well with Kozak’s rule. We found a lipase consensus motif with a putative catalytic serine (Prosite ID, PS00120), suggesting that PGAP1 is the deacylase (Fig. 3B).
FIG. 3. Cloning and characterization of PGAP1 cDNA. A, restoration of PI-PLC sensitivity. C10 cells were stably transfected with cDNAs of PGAP1, and a rescued cell line (RD3 cells) was established. The PI-PLC sensitivities of C10 mutant cells (a), RD3 rescued cells (b), and 3B2A wild-type cells (c) were assessed. B, alignment of sequences of PGAP1 homologues. The predicted amino acid sequences of rat (upper) and human (middle) PGAP1, and S. cerevisiae Bst1p (bottom) were aligned using the ClustalW software. Underlined regions, putative transmembrane domains predicted by the TMHMM program; bold gray line, lipase consensus domain; asterisk, putative catalytic serine. C, hydropathy plot of rat PGAP1. A hydropathy plot of PGAP1 was according to the Kyte and Doolittle program (31).
hydropathy profile of the predicted amino acid sequence suggested that PGAP1 has multiple transmembrane regions (Fig. 3C). Analysis by using a TMHMM program predicted six transmembrane regions (Fig. 3B). The lipase motif resided in a large hydrophilic region after the first transmembrane domain near the N terminus.

Regarding the human homologue, we found that the sequence of the first 591 amino acids of the hypothetical protein FLJ12377 was 90% identical with that of PGAP1. However, the predicted sequence of the C-terminal portion was not similar to PGAP1, and the entire sequence was shorter than that of PGAP1. The C-terminal sequence of FLJ12377 was found in genomic sequence of human PGAP1 gene (NCBI data base), suggesting that it was a product of alternative splicing. We also found ESTs whose sequences are homologous to the C-terminal portion of PGAP1. To obtain the full-length sequence of the human homologue, we amplified it from a human cDNA library by PCR and determined the sequence (DDBJ/GenBankTM/EMBL accession number AB128038). The identity of the predicted amino acid sequences of human and rat PGAP1 was 90%, and their lipase consensus sequences were identical (Fig. 3B).

*S. cerevisiae* *Bst1p* Is an Orthologue of PGAP1—We searched the data bases for homologues of PGAP1 and found an *S. cerevisiae* homologue of PGAP1 that was known as BST1 (Fig. 3B, DDBJ/GenBankTM/EMBL accession NC_001138) (18, 19). The amino acid identity of rat PGAP1 and yeast Bst1p was 18%, and the lipase consensus sequence was conserved. To examine whether BST1 was a functional homologue, we transiently transfected C10 mutant cells with BST1 cDNA and tested the PI-PLC sensitivity. We found a partial restoration of the PI-PLC sensitivity of the transfectants, indicating that Bst1p is an orthologue of PGAP1 (Fig. 4b).

ER Localization and Membrane Orientation of PGAP1 Protein—We fused FLAG or GST at the N terminus of PGAP1, and we examined whether they restore the PI-PLC sensitivity of C10 mutant cells. Both FLAG- and GST-tagged PGAP1 restored the deacylase activity (data not shown). To determine the intracellular localization of PGAP1, we transfected CHO cells with *FLAG-PGAP1* cDNAs, fractionated the cell lysate by sucrose density gradient ultracentrifugation, and quantified the activities of membrane marker enzymes. The plasma membrane and Golgi markers were fractionated into fractions 1 and 2 (Fig. 5B), and the ER marker was fractionated into fractions 3–5 (Fig. 5C). *FLAG-PGAP1* was determined by SDS-PAGE/Western blotting and showed a similar distribution to the ER marker (Fig. 5C). Thus, PGAP1 is an ER resident protein.

We determined the membrane orientation of PGAP1. Because the acyl group on the inositol moiety of GPI is oriented toward the ER lumen, if PGAP1 is the deacylase, the lipase motif would be oriented to the luminal side. If this is true, the N terminus of PGAP1 would be found on the cytoplasmic side of the ER. To examine this, we transiently transfected *GST-PGAP1* cDNAs into CHO cells (Fig. 6A), treated the cells with digitonin for selective permeabilization of the plasma membrane or with Triton X-100 for permeabilization of both the plasma ER membranes, and stained the cells for the GST tag (green) and BiP (red). To confirm the reliability of the experiment, we used PIG-M, which was double-tagged with GST and FLAG at the N terminus. The cytoplasmic orientation of the GST-FLAG-tag on PIG-M was established previously (13). The GST tag of PGAP1 was detected in the digitonin-treated cells (Fig. 6A-a), and the staining pattern and intensity were not significantly changed when cells were permeabilized with Triton X-100 (Fig. 6A-c). In contrast, BiP was only stained after treatment with Triton X-100 as expected (Fig. 6A, b and d). The staining profile of the GST-FLAG-PIG-M transfectants was similar to that of the GST-PGAP1 transfectants (Fig. 6B).
indicating that the N terminus of PGAP1 resides on the cytoplasmic side and that the lipase motif is oriented to the luminal side of the ER.

Site-directed Mutagenesis of a Putative Catalytic Serine—To address whether the lipase motif in PGAP1 is functional, we substituted the putative catalytic serine 174 with alanine (termed PGAP1(S174A)). We transiently transfected C10 mutant CHO cells with FLAG-tagged wild-type or S174A mutant of PGAP1. FLAG-ALDH cDNA was co-transfected to normalize the transfection efficiency. FLAG-tagged proteins were immunoprecipitated from cell lysates using anti-FLAG beads and analyzed by SDS-PAGE/Western blotting with monoclonal anti-FLAG antibodies. B, PI-PLC sensitivity of surface CD59 on C10 mutant CHO cells transfected with the S174A mutant of PGAP1. C10 cells were transfected with an empty vector (b), the wild-type (c), or S174A mutant (d) of FLAG-PGAP1. Cells were incubated with (shaded) or without (boldface outline) PI-PLC and stained for CD59. As a negative control, cells were stained with the second antibody only (dotted outline).

The Inositol Deacylation Is Important for the ER-to-Golgi Transport of GPI-APs—We investigated the role of the inositol deacylation of GPI-APs. The observation that an immature form of FLAG-CD59 accumulated in C10 cells (band b in Fig. 1B) suggested that the inositol deacylation was important for the maturation of GPI-APs. To test this idea, we analyzed the maturation of DAF using a pulse-chase labeling experiment. The ER form of DAF (~45 kDa) is transported to the Golgi and accepts multiple O-glycans and modification of the N-glycan to
become mature DAF (75 kDa). In C10 mutant cells, the amount of mature DAF gradually increased with a slow decrease in the amount of the ER form of DAF during the chase period (Fig. 8A, lanes 1–5). In contrast, in RD3 rescued cells, the increase in the mature DAF and decrease in the ER form of DAF were faster than those in C10 mutant cells (lanes 6–10). Parental 3B2A cells showed basically the same kinetics as RD3 cells (data not shown). Quantification of the data showed that the C10 mutant cells had a clear delay in the maturation of DAF; half the DAF matured within 20 min in RD3 rescued cells, while 60 min was required in C10 cells (Fig. 8B), indicating that the defect in the inositol deacylation affects the maturation of DAF. The molecular sizes of the mature DAF in C10 and RD3 cells were similar. Therefore, the extent of the glycosylation was not affected. We then analyzed total DAF in the whole cell lysates by SDS-PAGE and found a significant accumulation of the ER form of DAF during the chase period (Fig. 8B).

These results indicate that the inositol deacylation is important for the maturation of GPI-APs. Maturation of DAF and CD59 requires the ER-to-Golgi transport followed by glycosylation in the Golgi. Because the extent of the glycosylation was not affected, we concluded that the inositol deacylation is important for the ER-to-Golgi transport of GPI-APs.

**DISCUSSION**

The biosynthesis of GPI-APs can be separated into two phases. In the first phase, the immediate precursor of the GPI-anchor is synthesized and attached to the proteins, resulting in the generation of precursor GPI-APs. In the second phase, both the GPI and protein portions of the precursor GPI-APs are modified during transport from their site of generation in the ER to the cell surface. More than 20 genes involved in the first phase have been cloned and characterized, whereas little is known about the genes involved in the second phase. We have reported a number of genes in the first phase as PIG (for phosphatidylinositol glycan) genes, such as PIG-A, PIG-B, etc. (20). Here we report the first mammalian gene involved in the second phase. Because the second phase consists of events occurring after the attachment of the GPI-anchor to proteins, we termed this gene **PGAP1**.

The major findings in the present study are as follows: 1) that **PGAP1** encodes an ER-associated GPI inositol-deacylase, thereby providing a molecular basis for the report that inositol deacylation occurs in the ER soon after the generation of GPI-APs (6); and 2) that inositol deacylation is important for efficient ER-to-Golgi transport of GPI-APs.

**A Role for Inositol Deacylation in the ER-to-Golgi Transport of GPI-APs**—In PGAP1-deficient mutant cells, the ER-to-Golgi transport of DAF as measured by the generation of O-glycosylated Golgi form of DAF was clearly delayed. The time necessary for 50% of DAF to mature was 20 min in wild-type cells, while it was 60 min in the mutant cells. In S. cerevisiae, the **BST1** gene is homologous to **PGAP1** (18). PI-PLC sensitivity in PGAP1-deficient CHO cells was partially restored by transfection of **BST1**, showing that Bst1p is a functional homologue of PGAP1. Consistent with this result, maturation of Gas1p, a GPI-anchored protein whose maturation is dependent upon the transport to the Golgi (21), was delayed in **bst1**-deleted yeast (19), like DAF in C10 mutant cells. **BST1** (Bypass of Sec Thirteen 1) gene was identified by screening mutations that suppress lethality caused by down-regulation of **SEC13** gene, a gene for a component of COPII vesicle coatmers (18). In **sec13** mutant cells, normally deacylated GPI-APs may not be incorporated into COPII vesicles and may accumulate in the ER, possibly around the zone where COPII vesicles are generated. The fact that the simultaneous defect in **BST1** rescued the lethality caused by down-regulation of **SEC13** suggested that inositol-acylated GPI-APs may have been transported from the ER via some alternative way.

Emp24p (22) and Erp1p (23), members of p24 family, are thought to be components of cargo receptors for GPI-anchored protein Gas1p in S. cerevisiae. Both **emp24** and **erp1** mutations are also **sec13** bypass (18, 23). Taken together with a fact that surface transport of GPI-APs is essential for growth of S. cerevisiae, it is suggested that association of GPI-APs with their cargo receptors that would cause accumulation of GPI-APs in the ER is involved in the lethal effect of **sec13** mutation. It is therefore possible that the molecular basis of **sec13** bypass by **bst1** mutation is that the acyl chain remaining on inositol of GPI-APs in **bst1** mutant yeast might interfere with the efficient association with the cargo receptors, resulting in a lack of accumulation of GPI-APs. Thus, Bst1p/PGAP1-mediated inositol deacylation may be required for efficient incorporation of GPI-APs into COPII transport vesicles through the association with the cargo receptors.

**GPI Inositol Deacylation in Trypanosoma brucei Versus Mammals/S. cerevisiae**—In mammalian cells and the yeast...
acylated. In bloodstream parasites, the direct GPI-anchor precursor is not acylated, whereas in procyclic form parasites, the direct precursor of the GPI-mannosyl-glucosaminyl-phosphatidylinositol is acylated. Once added, the acyl chain remains until transfer of GPI to proteins. In African trypanosomes, the acceptor substrate for these acyltransferases is glucosaminyl-phosphatidylinositol. How the acyl chain is added one step later, because it is first added to mannosyl-glucosaminyl-phosphatidylinositol. However, the palmitoyl chain can be removed and added again at any of the later steps. The direct precursor of the GPI-anchor in bloodstream parasites is not acylated, whereas in procyclic form parasites, the direct GPI-anchor precursor is acylated. GPIdeAc gene that partially accounts for inositol deacylation activity in T. brucei has been cloned. GPIdeAc consisting of 558 amino acids has an N-terminal signal peptide but not a transmembrane domain. It is homologous to mammalian acylxyacyl hydrolase that removes fatty acids from bacterial lipopolysaccharides. GPIdeAc is much smaller than PGAP1 and has no significant sequence homology with PGAP1. GPIdeAc is much smaller than PGAP1 and has no significant sequence homology with PGAP1. GPIdeAc is hydrophilic whereas PGAP1 has multiple transmembrane domains. Therefore, GPIdeAc and PGAP1 are not homologous.

We found a sequence that has significant homology with PGAP1 in the T. brucei genome data base. Cloning and knock out or knock down of this gene are required to determine whether it is an inositol deacylase that accounts for the inositol deacylation activity remaining in the GPIdeAc knock out T. brucei (30).

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S. cerevisiae, an acyl chain is added to the 2-position of inositol early in the GPI biosynthetic pathway (24). The acylation is mediated by PIG-W in mammalian cells and by Bwt1p, the PIG-W orthologue, in S. cerevisiae (25). The acceptor substrate for these acyltransferases is glucosaminyl-phosphatidylinositol (26, 27). Once added, the acyl chain remains until transfer of GPI to proteins. In African trypanosomes, T. brucei, inositol acylation and deacylation reactions are more complex, differ from those in mammalian and yeast systems, and differ between the bloodstream and procyclic forms. In T. brucei, the acyl chain is added one step later, because it is first added to mannosyl-glucosaminyl-phosphatidylinositol (28, 29). However, the palmitoyl chain can be removed and added again at any of the later steps (28). The direct precursor of the GPI-anchor in bloodstream parasites is not acylated, whereas in procyclic form parasites, the direct GPI-anchor precursor is acylated. GPIdeAc gene that partially accounts for inositol deacylation activity in T. brucei has been cloned. GPIdeAc consisting of 558 amino acids has an N-terminal signal peptide but not a transmembrane domain. It is homologous to mammalian acylxyacyl hydrolase that removes fatty acids from bacterial lipopolysaccharides. GPIdeAc is much smaller than PGAP1 and has no significant sequence homology with PGAP1. GPIdeAc is hydrophilic whereas PGAP1 has multiple transmembrane domains. Therefore, GPIdeAc and PGAP1 are not homologous.

We found a sequence that has significant homology with PGAP1 in the T. brucei genome data base. Cloning and knock out or knock down of this gene are required to determine whether it is an inositol deacylase that accounts for the inositol deacylation activity remaining in the GPIdeAc knock out T. brucei (30).
Inositol Deacylation of Glycosylphosphatidylinositol-anchored Proteins Is Mediated by Mammalian PGAP1 and Yeast Bst1p
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