

Mechanism for Release of Alkaline Phosphatase Caused by Glycosylphosphatidylinositol Deficiency in Patients with Hyperphosphatasia Mental Retardation Syndrome*

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Background: Hyperphosphatasia was observed with glycosylphosphatidylinositol (GPI) deficiency due to mutation in *PIGV*, but not the *PIGM* gene.

Results: Alkaline phosphatase was released from cells defective in late-stage GPI biosynthesis.

Conclusion: In the presence of mannose-bearing GPI intermediates, GPI transamidase cleaves the C-terminal hydrophobic signal peptide and releases alkaline phosphatase.

Significance: This study explains the mechanism for release of anchorless GPI-anchored protein.

Hyperphosphatasia mental retardation syndrome (HPMR), an autosomal recessive disease characterized by mental retardation and elevated serum alkaline phosphatase (ALP) levels, is caused by mutations in the coding region of the phosphatidylinositol glycan anchor biosynthesis, class V (*PIGV*) gene, the product of which is a mannosyltransferase essential for glycosylphosphatidylinositol (GPI) biosynthesis. Mutations found in four families caused amino acid substitutions A341E, A341V, Q256K, and H385P, which drastically decreased expression of the *PIGV* protein. Hyperphosphatasia resulted from secretion of ALP, a GPI-anchored protein normally expressed on the cell surface, into serum due to *PIGV* deficiency. In contrast, a previously reported *PIGM* deficiency, in which there is a defect in the transfer of the first mannose, does not result in hyperphosphatasia. To provide insights into the mechanism of ALP secretion in HPMR patients, we took advantage of CHO cell mutants that are defective in various steps of GPI biosynthesis. Secretion of ALP requires GPI transamidase, which in normal cells, cleaves the C-terminal GPI attachment signal peptide and replaces it with GPI. The GPI-anchored protein was secreted substantially into medium from *PIGV*⁻, *PIGB*⁻, and *PIGF*-deficient CHO cells, in which incomplete GPI bearing mannose was accumulated. In contrast, ALP was degraded in *PIGL*⁻, *DPM2*⁻, or *PIGX*-deficient CHO cells, in which incomplete shorter GPIs that lacked mannose were accumulated. Our results suggest that GPI transamidase recognizes incomplete GPI bearing mannose and cleaves a hydrophobic signal peptide, resulting in secretion

of soluble ALP. These results explain the molecular mechanism of hyperphosphatasia in HPMR.

Recent whole exome sequencing of three siblings of nonconsanguineous parents demonstrated *PIGV* mutations in hyperphosphatasia mental retardation syndrome (HPMR),² an autosomal recessive disease characterized by mental retardation and elevated serum alkaline phosphatase (ALP) levels (1); *PIGV* is the second mannosyltransferase essential for glycosylphosphatidylinositol (GPI) biosynthesis (2). We have previously investigated the HPMR-associated *PIGV* mutation, A341E, and shown that the mutant *PIGV* protein was unstable and the mutant cDNAs restored only subnormal GPI biosynthetic activity after transfection into *PIGV*-deficient Chinese hamster ovary (CHO) cells (1).

Although ALP, a GPI-anchored protein, is present in all tissues throughout the entire body, it is present at a particularly high concentration in liver, bile duct, kidney, bone, and the placenta. Three isozymes, ALPI (intestinal), ALPL (tissue non-specific; liver/bone/kidney), and PLAP (placental, also termed ALPP) are known in humans; the elevated ALP in HPMR is ALPL (3).

The GPI anchor backbone is synthesized in the endoplasmic reticulum (ER) and transferred to proteins that have a GPI attachment signal at the C terminus (hereafter referred to as proproteins) by GPI transamidase complex. The GPI transamidase consists of five proteins: *PIGK*, *Gaa1*, *PIGS*, *PIGT*, and *PIGU* (4, 5). The GPI attachment signal sequence is a triplet of

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² The abbreviations used are: HPMR, hyperphosphatasia mental retardation syndrome; ALP, alkaline phosphatase; GPI-AP, glycosylphosphatidylinositol-anchored protein; PLAP, placental alkaline phosphatase; ALP, alkaline phosphatase; DAF, decay accelerating factor; ER, endoplasmic reticulum; LC/ESI, liquid chromatography/electrospray ionization; *PIGV*, phosphatidylinositol glycan class V.

small amino acids (termed ω , $\omega+1$, and $\omega+2$), a hydrophilic spacer of 7–10 amino acids and C-terminal hydrophobic stretch (6). The GPI transamidase cleaves peptide bond between the ω and $\omega+1$ residues and attaches GPI to the C terminus of the ω site amino acid.

Cell-surface GPI-anchored proteins (GPI-APs) can be released by mechanisms such as cleavage by GPI-specific phospholipases or other enzymes targeting GPI glycan or the peptide part, and shedding intact GPI-APs (7). In fact, many GPI-APs such as ALP, CD87/urokinase-type plasminogen activator receptor, and CD14 that are found in serum are most likely derived from those expressed as GPI-APs on the cell surface (8, 9). When proproteins are not modified by GPI, due either to defective GPI biosynthesis or GPI transamidase, they are retained in the ER, leading to two possible outcomes: intracellular degradation or secretion (10). Serum ALP levels in PIGV-defective patients are significantly elevated, with decreased surface expression (1, 3). Hyperphosphatasia is not observed in patients with inherited GPI deficiency due to PIGM mutation (Table 1) (11). PIGM deficiency is caused by a mutation in the *PIGM* gene promoter region that encodes the first mannosyltransferase, which is also essential for GPI biosynthesis (12). To explain this difference, we considered the possibility that the pathogenesis of hyperphosphatasia is critically dependent on the step of GPI biosynthesis that is defective. Using mutant CHO cells that are defective in various GPI biosynthesis steps, we show that secretion of ALP requires GPI transamidase, which is activated by GPI bearing at least one mannose.

EXPERIMENTAL PROCEDURES

Cells and Culture—GPI-deficient CHO cells, PIGL⁻ (M2S2), PIGX⁻ (2.37), DPM2⁻ (B5), PIGV⁻ (D9PA15.6), PIGB⁻ (1.33), PIGF⁻ (3.37), PIGK⁻ (10.2.2), and PIGU⁻ (C311PA16) are previously described (13). These mutant CHO cells express human CD59 and decay accelerating factor (DAF). These mutant CHO cells and CHO-K1 wild-type cells were cultured in Ham's F-12 medium (Sigma) supplemented with 10% fetal calf serum (FCS).

Transfection of Plasmids into PIGV-deficient CHO Cells—FLAG-tagged human *PIGV* cDNA and its mutants generated by site-directed mutagenesis were subcloned into pTA, a weak expression plasmid with minimal TATA box promoter or pME, a strong expression plasmid with SR α promoter (14). Plasmids were transfected by electroporation into PIGV-deficient CHO cells. Cells (5×10^6) were suspended in 0.4 ml of Opti-MEM and electroporated with 10 μ g each of the plasmids at 260 V and 960 microfarads using a Gene Pulser (Bio-Rad). Levels of PIGV protein in cells were determined by Western blotting. Band intensities of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls. For transfection of *PLAP* cDNA into PIGV-deficient and PIGV-rescued CHO cells, pME HA-PLAP plasmid and pME luciferase were cotransfected into PIGV-deficient CHO cells together with pME PIGV or empty pME vector. In pME HA-PLAP for expression of HA-tagged human PLAP, the ω site aspartic acid was changed to serine for more efficient processing by GPI transamidase (15). To generate an ω site mutant that cannot be

processed by GPI transamidase, aspartic acid was changed to proline (15).

FACS Analyses for CD59 and PLAP—Surface expression of GPI-APs, CD59, and PLAP was determined by staining cells with mouse anti-CD59 (5H8) or mouse anti-PLAP (8H6, Sigma) antibodies, followed by a phycoerythrin-conjugated anti-mouse IgG antibody and analyzed by flow cytometer (Cant II; BD Biosciences, Franklin Lakes, NJ) using FlowJo software (Tommy Digital Inc., Tokyo, Japan).

Pulse-Chase Metabolic Labeling—Cells (2×10^6) plated on 6-well plates were preincubated for 30 min at 37 °C in methionine- and cysteine-free DMEM (Invitrogen) containing 10% dialyzed FCS, pulsed with 100 μ Ci/ml of [³⁵S]methionine and [³⁵S]cysteine (EXPRE³⁵S³⁵S Protein Labeling Mix, PerkinElmer Life Sciences) for 10 min at 37 °C, and then chased after adding cold 0.7 mM methionine and 0.3 mM cysteine. After each chase period, the culture medium and cell lysates were prepared, and incubated with a monoclonal anti-DAF antibody (IA10) and protein A-Sepharose (GE Healthcare) that had been precoated with a nonlabeled CHO-K1 cell lysate. Immunoprecipitates were separated by SDS-PAGE and detected using a BAS 1000 analyzer (Fujifilm, Tokyo, Japan).

Liquid Chromatography/Electrospray Ionization/Tandem Mass Spectrometry (LC/ESI MS/MS) Analysis of PLAP Secreted from PIGV-deficient CHO Cells—Using human PLAP expressing vector pME HA-PLAP with ω site serine, we changed alanine at 495 to lysine to have a shorter C-terminal peptide after digestion by trypsin for easier detection in mass spectrometry. PIGV-deficient CHO cells were permanently transfected with the modified pME HA-PLAP, and released HA-PLAP was captured from the culture medium by anti-HA beads (Sigma) after 2 days and subjected to SDS-PAGE and extracted after in-gel digestion with trypsin for LC/ESI MS/MS analysis using ADVANCE UHPLC (Michrom Bioresources, Auburn, CA) and LTQ Orbitrap Velos (Thermo Scientific, Waltham, MA).

Measurement of ALP Activity—For transient transfection experiments, 0.2 μ g of *PLAP* cDNA was lipofected together with 0.6 μ g of cDNA responsible for each GPI biosynthesis mutant for rescue or empty pME as controls, and 0.016 μ g of pME luciferase for normalization using Lipofectamine 2000 (Invitrogen). Media were changed the next day; ALP activities in cell lysates and culture media were measured on the following day using the SEAP assay kit (Clontec, Madison, WI). Luciferase activities of cell lysates were measured using the Luciferase assay kit (Promega, Madison, WI) for the normalization of gene expression. Relative activity in culture medium of each mutant was expressed as the ratio of normalized ALP activity in the culture medium against the normalized total ALP activity of each rescued cell.

RESULTS

PIGV Mutations Found in Patients with HPMPR-destabilized PIGV Protein—Nucleotide sequencing revealed the homozygous or compound heterozygous *PIGV* mutations in each family: A341E/A341E in family A, H385P/A341V in family B, Q256K/Q256K in family C, A341E/A341V in family D, and A341W/A341W in family E (1). All mutations were positioned within the transmembrane region of PIGV (Fig. 1A).

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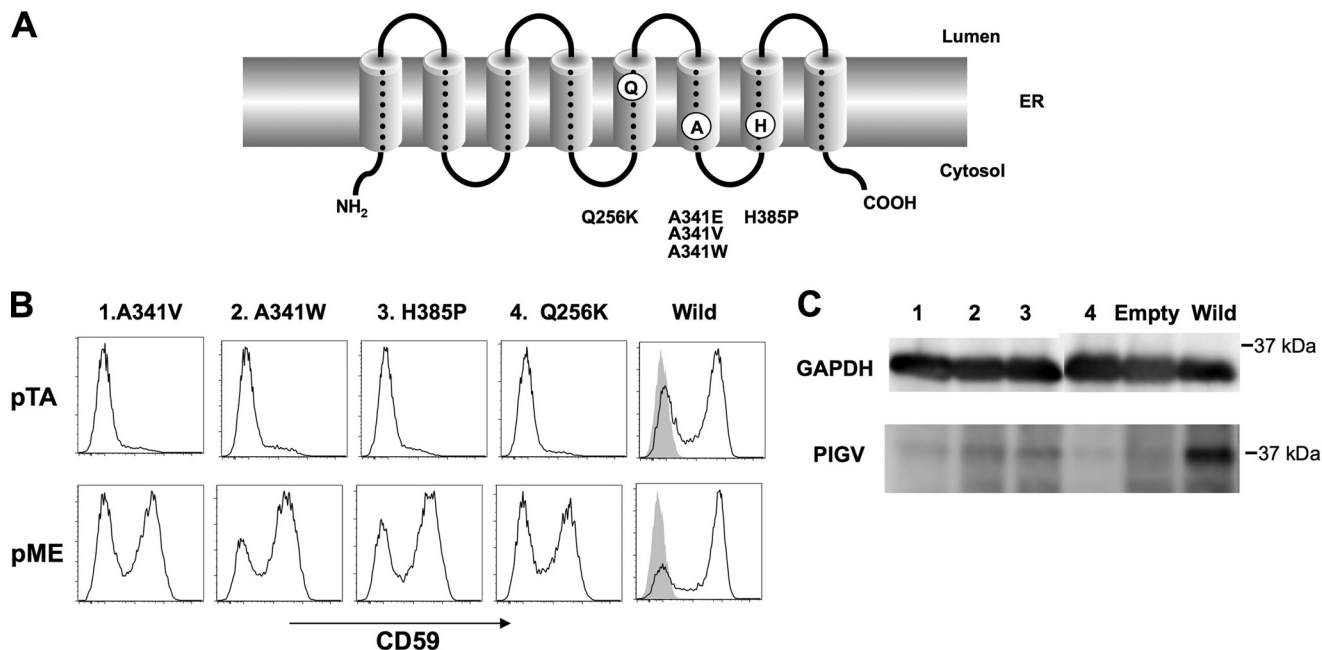


FIGURE 1. *A*, PIGV mutations found in patients with HPMR syndrome. *B*, inefficient restoration of GPI-AP expression on PIGV-deficient CHO cells by mutant PIGV cDNA. PIGV-deficient CHO cells were transiently transfected with weak promoter-driven pTA PIGV constructs (*upper panel*) or strong promoter-driven pME PIGV constructs (*lower panel*) carrying indicated mutations or a wild-type construct or an empty vector (*gray shadow*) and were analyzed for the surface expression of CD59 by FACS. *C*, decreased expression of mutant PIGV. Expressions of FLAG-tagged PIGV protein were analyzed by Western blotting 2 days after transfection with strong promoter-driven pME PIGV constructs. Number of each *lane* indicates each mutant shown in *B*; GAPDH is a loading control.

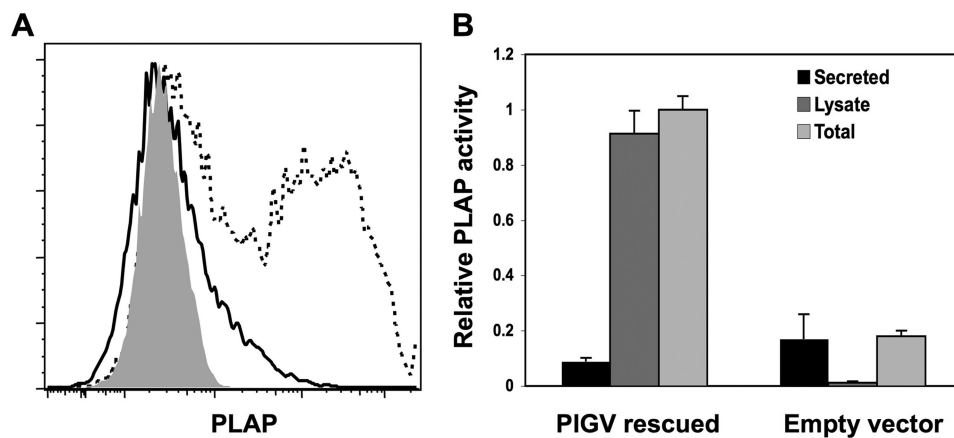


FIGURE 2. *A*, surface expression of PLAP after cotransfection with PIGV into PIGV-deficient CHO cells. PIGV-deficient CHO cells were transiently transfected with pME HA-PLAP together with pME PIGV (*dotted line*) or empty vector (*solid line*) and analyzed for the surface expression of PLAP by FACS. *Shadow*, without PLAP expression vector. *B*, ALP activity in culture medium and cell lysate after cotransfection of PLAP and PIGV cDNAs into PIGV-deficient CHO cells. Each transfected in *A* was analyzed. Relative ALP activity in culture medium (*black bars*), cell lysates (*dark gray bars*), and total (*light gray bars*) against the total ALP activity in PIGV-rescued CHO cell. Each value indicates the mean \pm S.D. of four independent experiments.

To analyze the activities of these mutant PIGV proteins, we transfected PIGV-deficient CHO cells with plasmids bearing mutant PIGV driven by the strong SR α promoter (pME) or by the weak promoter containing only a TATA box (pTA) and analyzed surface expression of CD59, a GPI-AP, by FACS. PIGV mutants driven by the strong promoter rescued the surface expression of CD59 almost completely, whereas those driven by the weak promoter failed to restore (Fig. 1*B*). Even in the cells rescued by the strong promoter-driven PIGV mutants, expression of the PIGV mutant proteins were drastically decreased compared with that of the wild-type (Fig. 1*C*). Taken together, our results show that single amino acid substitutions within the transmembrane region of the PIGV protein destabilized this protein.

Proteins Are Cleaved at ω Site and Released from PIGV-deficient Cells, in a PIGK-dependent Manner—Patients with PIGV deficiency have elevated serum ALP levels, despite the decreased surface expression (1). A similar observation was seen in PIGV-deficient CHO cells (Fig. 2). After transient transfection with PLAP cDNA, only very low surface expression of PLAP was seen in PIGV-deficient cells, whereas much higher expression was seen on PIGV-rescued cells (Fig. 2*A*, *solid versus broken lines*). In parallel with the profile of surface PLAP expression, very little activity was found in lysates of PIGV-deficient cells, whereas high PLAP activity was found in lysates of PIGV-rescued cells (Fig. 2*B*, *dark gray bars*). In contrast, a substantially higher amount of PLAP was secreted into the culture medium from PIGV-deficient

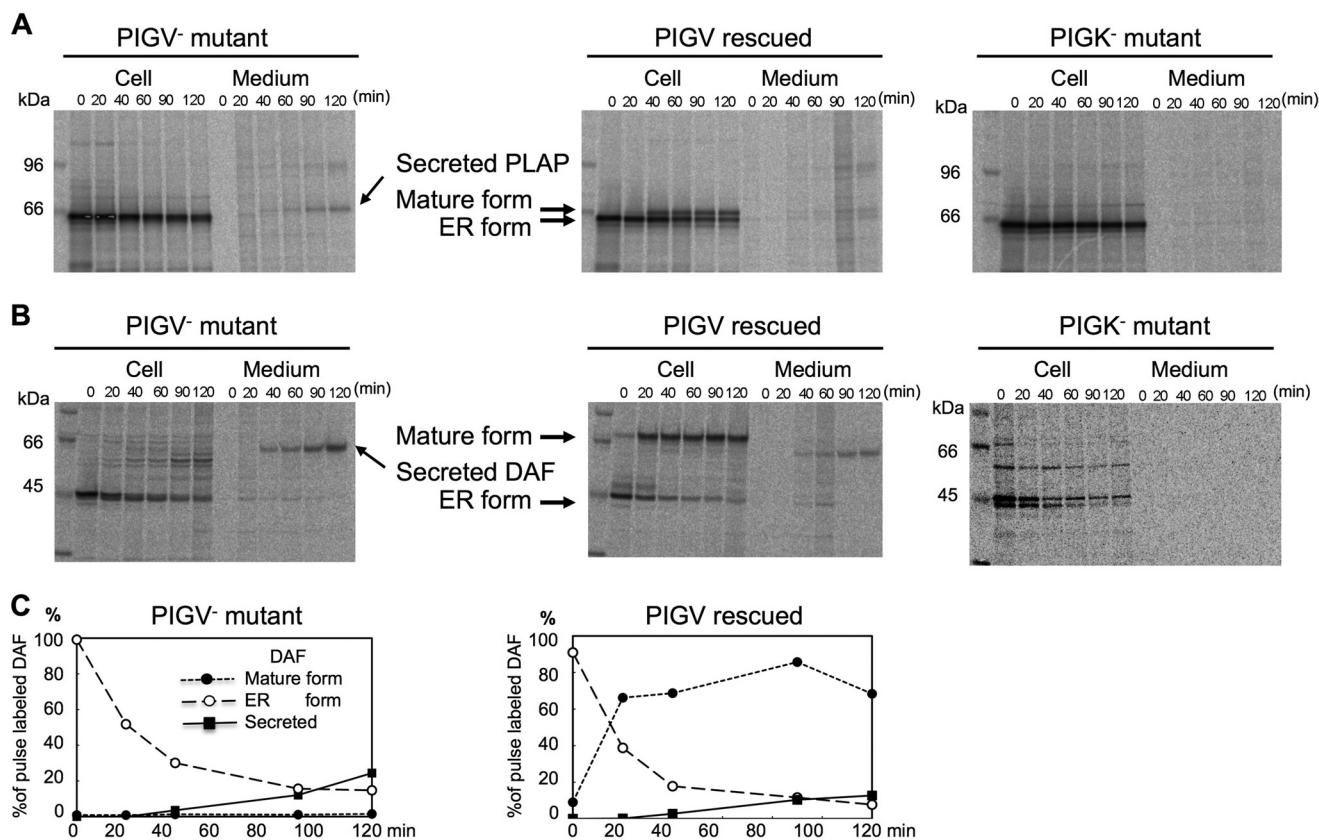


FIGURE 3. Pulse-chase metabolic labeling of GPI-APs. *A*, HA-PLAP expressing PIGV-deficient cells (*left panel*), PIGV-rescued (*middle panel*) and PIGK-deficient (*right panel*) CHO cells were pulsed with [³⁵S]methionine and [³⁵S]cysteine for 10 min and then chased for the indicated periods. The cell lysates and culture supernatants were immunoprecipitated with an anti-HA antibody, separated by SDS-PAGE, and analyzed using a BAS 1000 analyzer. *B*, DAF expressing PIGV-deficient cells (*left panel*), PIGV-rescued (*middle panel*), and PIGK-deficient (*right panel*) CHO cells were analyzed as in *A*. *C*, quantified data of *B*. Intensity of total labeled protein at the start of the chase was 100%. Broken lines, ER form; solid lines, secreted DAF; dotted lines, mature form.

TABLE 1

Serum ALP activities in two different GPI deficiencies

Total ALP in serum ^a (n) ^b	PIGM deficiency	Patient 1	Patient 2	Patient 3 ^d			
			0.47 (2)	0.46 (2)	1.3 (1)		
	PIGV deficiency ^c	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
		2.7 (2)	2.2 (1)	10.7 ± 7.8 (4)	1.3 ± 0.6 (7)	2.5 (1)	2.0 (1)

^a ALP activity (a ratio of the activity in the patient's serum to that in the age-specific upper limit of normal serum).

^b Number of measurements.

^c Ref. 3.

^d Died from portal vein thrombosis.

mutant cells than from PIGV-rescued cells (Fig. 2*B*, black bars).

To gain insight into the mechanism, we analyzed the kinetics of secretion and degradation of PLAP and DAF, another GPI-AP, in PIGV-deficient and -rescued cells, using pulse-chase metabolic labeling experiments. PLAP is *N*-glycosylated and DAF is highly *O*-glycosylated during maturation in the Golgi, such that each ER form can be distinguished from the mature form by their molecular sizes. In the PIGV-rescued cells, PLAP is gradually matured with glycosylation; a small amount of non-glycosylated and glycosylated forms could be detected in the medium (Fig. 3*A*, middle panel). In the PIGV-mutant cells, the glycosylated form could not be detected in the cell lysates but a significant amount of it was secreted into the medium (Fig. 3*A*, left panel). This secretion was not observed in PIGK-deficient

cells at all, suggesting that secretion required GPI transamidase activity (Fig. 3*A*, right panel). Similarly, in the PIGV-rescued cells, DAF was matured with glycosylation and a small amount of glycosylated DAF could be detected in the medium (Fig. 3*B*, middle panel). The size of secreted DAF was smaller than that of the mature form in the cell lysate, presumably due to partial loss of sialic acid after secretion (16). In the PIGV-mutant cells, the glycosylated DAF form could not be detected in the lysates but a significant amount of it was secreted into the medium (Fig. 3*B*, left panel). This secretion was completely absent in the PIGK-deficient cells (Fig. 3*B*, right panel). Quantified data showed that, in PIGV-deficient cells, about 25% of total pulse-labeled DAF was secreted into the medium, about 15% remained as the ER form and 60% was degraded, whereas about 14% of total labeled DAF was secreted in PIGV-rescued cells

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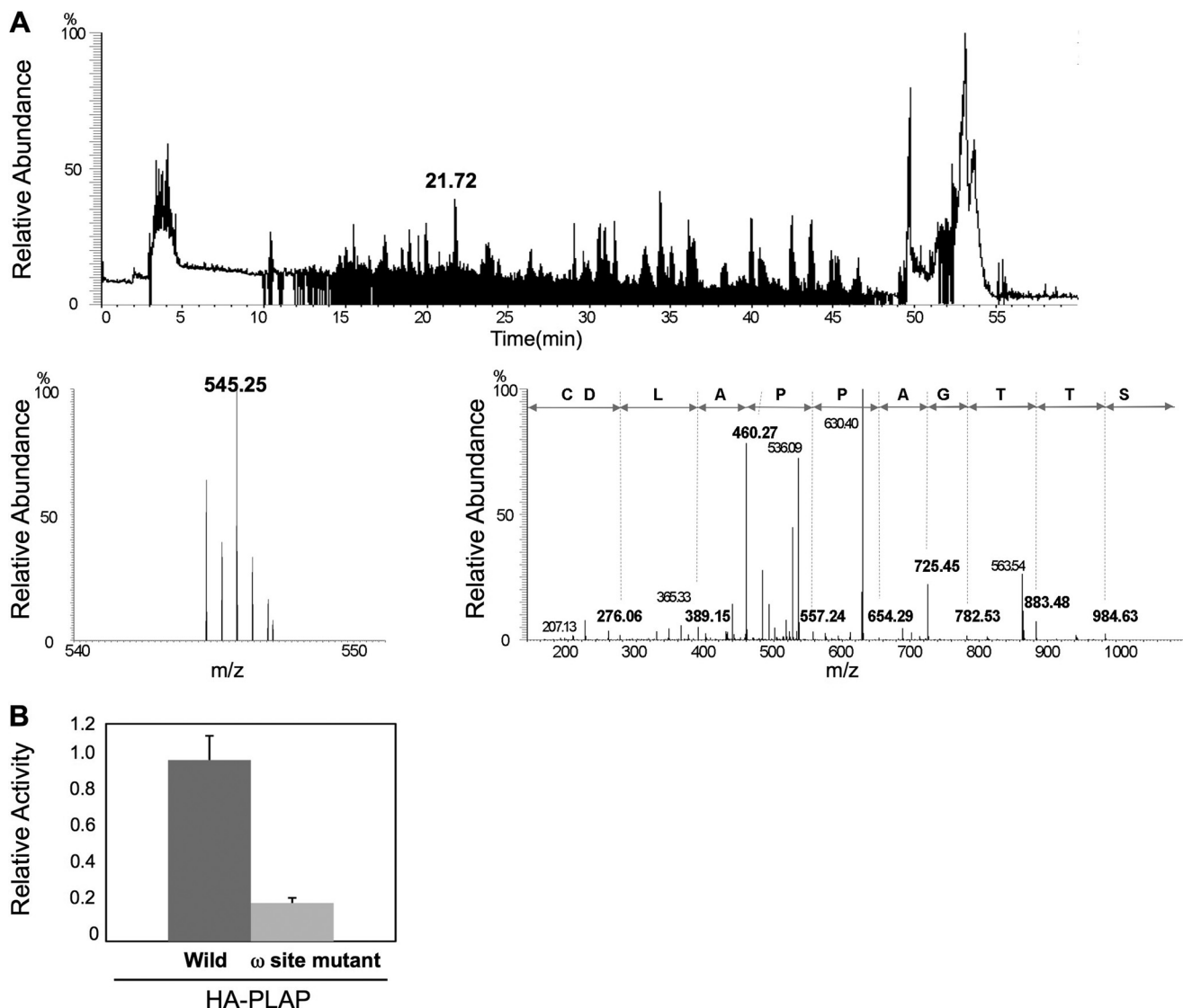


FIGURE 4. *A*, identification and characterization of C-terminal peptide by LC/ESI MS/MS. *Top*, spectra of peptides eluted from LC. The peak at the retention time of 21.72 min contained a C-terminal peptide cleaved at the ω site in the GPI attachment signal; *bottom left*, the first MS analysis of the peptides eluted at 21.72 min; *bottom right*, MS/MS spectrum of a parent ion with m/z 545.25²⁺ and its sequence. The *b*-series fragments are indicated in *bold*. *B*, the ω site-dependent release of PLAP from PIGV-deficient CHO cells. PIGV-deficient CHO cells were transiently cotransfected with wild-type (dark gray bar) or ω site mutant (light gray bar) HA-PLAP and luciferase reporter construct for normalization. Relative PLAP activities in the media are shown.

and about 70% was in the matured form (Fig. 3C). These results are consistent with the fact that ALP serum levels from PIGV-deficient patients are about two times higher than normal values (Table 1). These results suggest that a significant amount of GPI-APs is secreted in the PIGV mutant cells in a transamidase-dependent manner.

To determine the cleavage site at the C-terminal part of the proprotein, we analyzed the structure of the secreted PLAP using LC/ESI MS/MS. We permanently transfected HA-tagged PLAP into PIGV-deficient CHO cells, purified HA-PLAP secreted from the cells into the culture medium, and analyzed its structure by LC/ESI MS/MS after trypsin digestion. Under high-peptide coverage (41 of 49 peptides identified), we found the fragment at a mass to charge ratio (m/z) of 545.25, corresponding to the C-terminal peptide CDLAPPAGTTTS, suggesting that PLAP was secreted by cleavage at the ω site in the GPI attachment signal (Fig. 4A).

To further confirm cleavage at the ω site, we mutated the ω site in PLAP cDNA from aspartic acid to proline, which makes the PLAP proprotein resistant to GPI transamidase cleavage (15). We transfected the wild-type and mutant PLAP cDNAs into PIGV-deficient CHO cells. We found that ALP activity in medium from the PIGV-deficient cell transfected with the mutant PLAP was drastically decreased, indicating that the release was the result of cleavage at the ω site of PLAP by the GPI transamidase in the ER (Fig. 4B).

Activation of GPI Transamidase Requires GPI Intermediate Bearing Mannose—We have so far shown that, in PIGV-deficient cells, GPI-APs are hydrolyzed by GPI transamidase in the absence of mature GPI. These observations are consistent with the fact that patients with PIGV deficiency show an elevated serum ALP level (1). In striking contrast, however, patients with PIGM deficiency, who also suffer from the absence of mature GPI, do not show elevated ALP levels (11). GPI transamidase

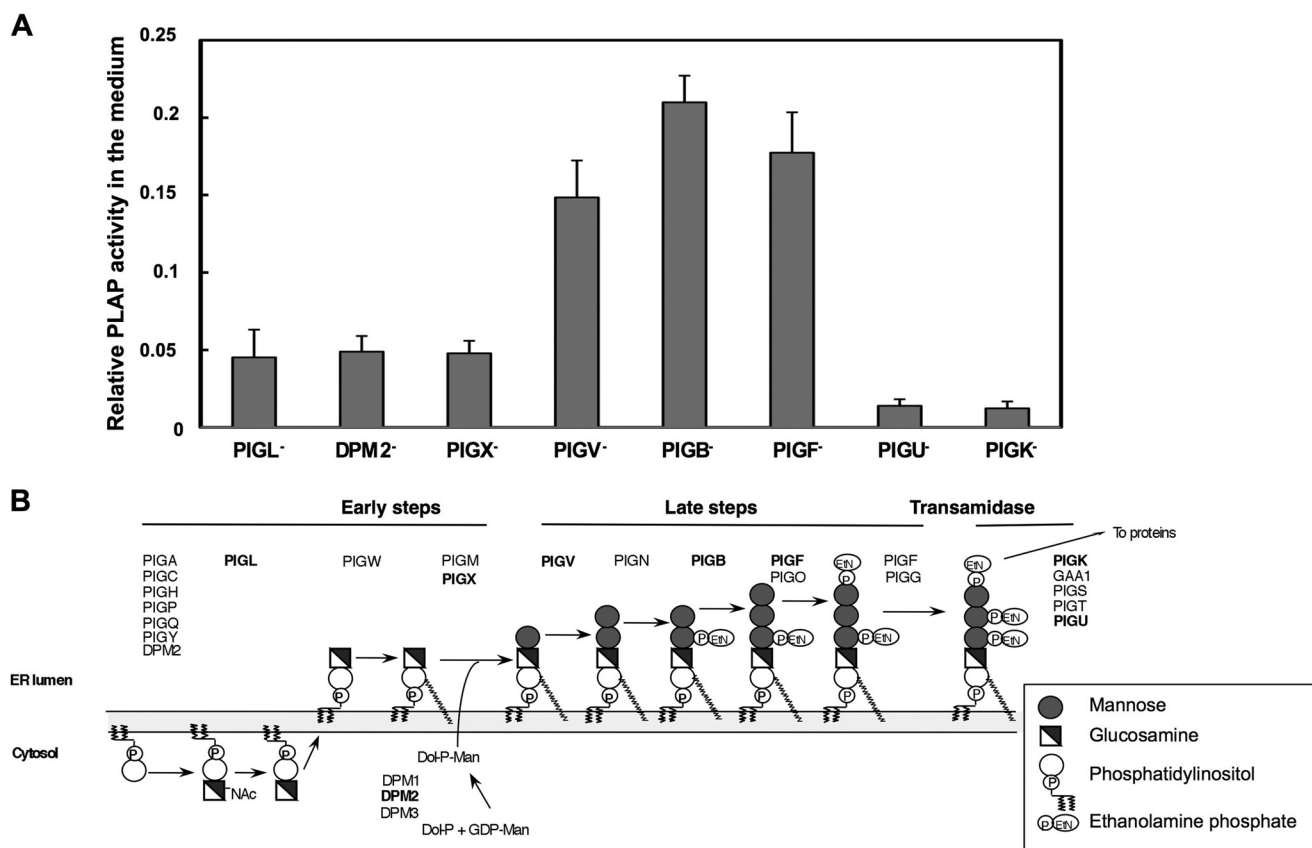


FIGURE 5. Secretion of PLAP from CHO cell mutants defective in various GPI biosynthesis steps. A, various CHO cell mutants were transiently transfected with PLAP expression vector and the luciferase reporter construct for normalization together with cDNA of each responsible gene or empty vector. Normalized relative PLAP activities in the medium from various mutants are shown. B, GPI biosynthesis pathway. Genes defective in CHO cell mutants used in A are shown in *bold*. Modified from Ref. 20.

recognizes a mature GPI and attaches its ethanolamine moiety to the C terminus of the proprotein after processing the GPI attachment signal (6). Cells from patients with PIGM deficiencies have functional transamidase, and yet they do not secrete GPI-APs. To explain the difference, we focused on the structure of GPI intermediates that accumulate in PIGM- and PIGV-deficient cells. Glucosaminyl-(inositol acylated) phosphatidylinositol (GlcN-acyl-PI) accumulates in PIGM-deficient cells, whereas mannosylated GlcN-acyl-PI accumulates in PIGV-deficient cells (Fig. 5B). We therefore hypothesized that efficient cleavage of the GPI attachment signal by GPI transamidase requires the GPI intermediate bearing the first mannose. We have a repertoire of GPI biosynthetic mutants that are defective in the early steps before PIGM and also in the late steps after PIGV. We transfected these various CHO cell mutants and their rescued cells with a PLAP expression vector and measured ALP activity in the medium. ALP activities in the medium were significantly higher in the group of late step mutants than in the group of early step mutants (Fig. 5A). GPI intermediates accumulated in the late step mutants (*PIGV*⁻, *PIGB*⁻, and *PIGF*⁻ cells) have mannoses, whereas those in the early step mutants (*PIGL*⁻, *DPM2*⁻, and *PIGX*⁻ cells) lack mannose, suggesting that GPI transamidase more efficiently cleaved the GPI attachment signal by recognition of mannose residue, leading to release of proproteins (Fig. 5, A and B). Only a trace PLAP activity was found in the group of *PIGK*⁻ and another GPI

transamidase mutant *PIGU*⁻, further supporting GPI transamidase-mediated ALP release.

DISCUSSION

In this study, we found that GPI transamidase plays a critical role in secretion of proproteins in the absence of mature GPI, as observed in *PIGV*-deficient HPMR syndrome, and that the presence of the mannose residue of immature GPI is critical for cleavage of the proproteins by GPI transamidase, which would clearly explain why serum ALP is not elevated in PIGM-deficient patients.

In wild-type cells, the GPI transamidase complex recognizes both the GPI attachment signal and mature GPI, and processes the proprotein at the ω site to attach GPI. GPI-APs are then expressed on the cell surface and a small fraction are released from the cell surface (Fig. 6, top). In late step mutants such as *PIGV*⁻, *PIGB*⁻, and *PIGF*⁻ deficient cells, whereas a major fraction of proproteins were degraded, a substantial fraction of proprotein was hydrolyzed at the ω site by the GPI transamidase, and was released into the culture medium (Fig. 6, second top). In contrast, in early step mutants such as *PIGL*⁻, *PIGX*⁻, and *DPM2*-deficient cells, GPI transamidase could not hydrolyze the proprotein, resulting in degradation of most proproteins (Fig. 6, second bottom). In transamidase mutants, proprotein was completely degraded within the cell (Fig. 6, bottom). GPI intermediates containing at least one mannose accumulated in

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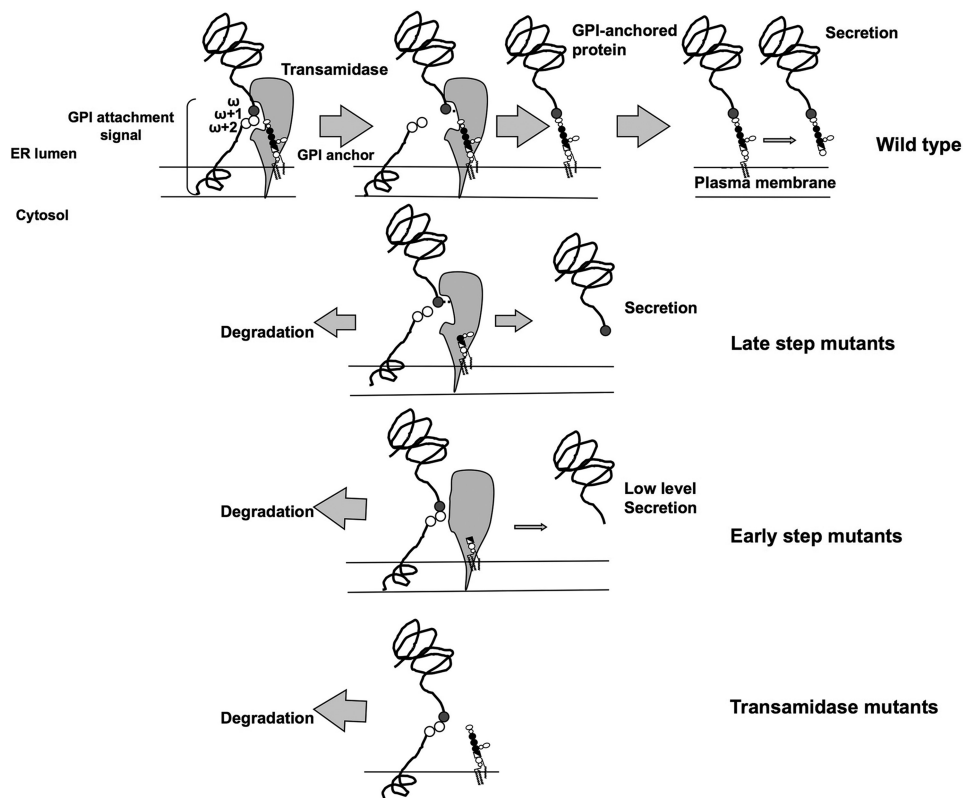


FIGURE 6. **Models of different processing of GPI-AP proprotein in wild-type and GPI-defective cells.** In wild-type cells, the GPI transamidase complex recognizes both the GPI attachment signal and GPI, and processes the proprotein at the ω site and attaches it to GPI. GPI-APs are expressed on the cell surface and a small fraction of GPI-AP is released by some GPI-cleaving enzyme (top). In late step mutants, a significant fraction of proprotein is processed at the ω site by the GPI transamidase, and is released into the culture medium, whereas the rest of the proprotein is degraded (second top). In early step mutants, GPI transamidase is not activated; bound proprotein is mostly degraded and a small fraction is released by an unknown mechanism (second bottom). In GPI transamidase mutants, proprotein is completely degraded within the cell (bottom).

the late step mutants, suggesting that the mannose residue of GPI intermediate is essential for proprotein processing by GPI transamidase. These results are consistent with the observed difference of the phenotype of the patients with PIGM and PIGV deficiencies.

The fact that at least one mannose residue is required for GPI transamidases to cleave the GPI attachment signal suggests the presence of a mannose-recognizing subunit in GPI transamidase, which is composed of five proteins. By homology search using PSI-BLAST, we found that PIGU has a region homologous to the putative catalytic loop of PIGM (*E* value, 8.57×10^{-6}) where PIGM should recognize the mannose residue of dolichol-phosphate mannose, although the putative catalytic aspartic acid residue of PIGM is not conserved in PIGU. Because Gaa1 is essential for the binding of mature GPI (17), PIGU might cooperate with Gaa1 to bind GPI and activate GPI transamidase by recognizing mannose.

Inherited PIGN deficiency was recently identified but was reported not to be associated with hyperphosphatasia (18). PIGN attaches an ethanolamine phosphate side branch to the first mannose, which is a common component in GPI-APs. A PIGN knock-out in embryonal carcinoma F9 cells showed only a partial decrease in surface expression of GPI-APs (19), which indicates that an aberrant GPI without the ethanolamine phosphate side branch was utilized for the production of GPI-APs. The efficiency for attachment of the aberrant GPI to propro-

teins in patients partially defective for PIGN might be less than normal but still significant, which could suppress the hydrolysis and release of proproteins. Based on our results, we predict that, in addition to PIGV mutations, defects in other GPI biosynthesis genes such as *PIGB*, *PIGO*, and *PIGF* may also cause HPMR syndrome. We also predict that hyperphosphatasia may not be seen with defects in early GPI biosynthesis genes such as *PIGL* and *PIGW*.

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