Review

Biosynthesis and deficiencies of glycosylphosphatidylinositol

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Abstract: At least 150 different human proteins are anchored to the outer leaflet of the plasma membrane via glycosylphosphatidylinositol (GPI). GPI preassembled in the endoplasmic reticulum is attached to the protein’s carboxyl-terminus as a post-translational modification by GPI transamidase. Twenty-two PIG (for Phosphatidylinositol Glycan) genes are involved in the biosynthesis and protein-attachment of GPI. After attachment to proteins, both lipid and glycan moieties of GPI are structurally remodeled in the endoplasmic reticulum and Golgi apparatus. Four PGAP (for Post GPI Attachment to Proteins) genes are involved in the remodeling of GPI. GPI-anchor deficiencies caused by somatic and germline mutations in the PIG and PGAP genes have been found and characterized. The characteristics of the 26 PIG and PGAP genes and the GPI deficiencies caused by mutations in these genes are reviewed.

Keywords: glycosylphosphatidylinositol, glycolipid, post-translational modification, somatic mutation, germline mutation, deficiency

Introduction

At least 150 different human proteins are post-translationally modified by glycosylphosphatidylinositol (GPI) at the carboxyl (C)-terminus. These proteins are expressed on the cell surface by being anchored to the outer leaflet of the plasma membrane via the phosphatidylinositol (PI) moiety and termed GPI-anchored proteins (GPI-APs). While the functions of GPI-APs are various, including hydrolytic enzymes, adhesion molecules, receptors, protease inhibitors, and complement regulatory proteins, they share similar characteristics based on their common glycolipid membrane anchors. GPI-APs are typical raft-associated proteins and tend to form homodimers. GPI-APs can be released from the cell after cleavage by GPI-cleaving enzymes or GPIases. These characteristics are critical for the functions of individual GPI-APs and for embryogenesis, neurogenesis, fertilization, and the immune system.

Protein-bound mammalian GPI-anchors have a core backbone, which can be modified by side branches (Fig. 1). The structure of the core backbone is EtNP-6Man1-2Man1-6Man1-4GlcN1-6myoInositol-phospholipid (where EtNP is ethanolamine phosphate, Man is mannose, and GlcN is glucosamine). EtNP linked to Man3, the 2-position of Man1, the 1-4-linked Man. Some of the mammalian GPI-APs have a fourth Man as a side branch that is 1,2-linked Man, makes an amide bond with the C-terminus of the protein. 1-Alkyl-2-acyl PI is the major form of inositol phospholipid in protein-bound mammalian GPI-APs and diacyl PI is a minor form. Mammalian GPI-APs usually have two saturated fatty chains, with a small fraction containing one unsaturated bond in an sn1-linked chain. The sn2-linked fatty acid is usually stearic acid (C18:0), while the sn1-linked chain has 18 or 16 carbons.

Mammalian GPI-APs have a common EtNP side branch linked to the 2-position of Man1, the α1-4-linked Man. Some of the mammalian GPI-APs have a fourth Man as a side branch that is α1-2-
linked to Man3. These side branches are added to GPI-precursors during biosynthesis in the endoplasmic reticulum (ER) before attachment to proteins. The third known side branch is N-acetylgalactosamine (GalNAc), which is further modified by galactose and sialic acid. Although the enzymes required for making the GalNAc-containing side branches remain to be identified, these modifications are likely to occur after attachment to proteins and after transport to the Golgi apparatus.

GPI precursors are synthesized in the ER and transferred en bloc to proteins that bear a GPI-attachment signal sequence at the C-terminus (Fig. 2). Transfer of GPI to proteins is mediated by GPI-transamidase, which recognizes the GPI-attachment signal sequence and replaces it with the GPI-precursor by a transamidation reaction (Fig. 3). After protein-attachment, GPI-APs are structurally and remodelled at both lipid and glycan parts of GPI, and finally expressed on the cell surface (Fig. 4).

At least 26 genes are involved in the biosynthesis, protein-attachment, and remodeling of mammalian GPI (Figs. 2 and 4). For the last two decades, my laboratory has been working on the identification and characterisation of these genes, and clarification of their genetic deficiencies caused by somatic and germline mutations in these genes. In this article, I review these genes and GPI deficiencies.

**PIG (for Phosphatidyl Inositol Glycan) genes involved in the biosynthesis and transfer of GPI**

1. **PIGA** gene. Kamitani and colleagues found that murine Ltk- cells were defective in the first step of GPI biosynthesis and belonged to the class H complementation group. They used an expression cloning system involving a cDNA library generated in a polyoma virus-based vector and identified a PIGA cDNA that could restore surface GPI-AP expression on Ltk- cells. Since the Ltk- cell line was transformed with Epstein-Barr virus (EBV) and expressed EBNA1 nuclear antigen, we established an expression cloning system in which JY5 cells were transfected with an EBV vector-based cDNA library. In this system, cells that regained surface expression of GPI-APs were collected by cell sorting, and episomal plasmids were rescued and retransfected into JY5 cells. This process was repeated three times and finally a PIGA cDNA that could restore surface GPI-AP expression on JY5 cells was cloned.

The PIG-A protein encoded by the PIGA cDNA consists of 484 amino acids, has one transmembrane domain near the C-terminus, and is expressed in the ER. The amino (N)-terminal large hydrophilic portion resides on the cytoplasmic side and a short hydrophilic portion resides in the ER lumen. PIG-A is a catalytic subunit of GPI-GlcNAc transferase (GPI-GnT).

2. **PIGH** gene. Kamitani and colleagues found that murine Ltk- cells were defective in the first step of GPI biosynthesis and belonged to the class H complementation group. They used an expression cloning system involving a cDNA library generated in a polyoma virus-based vector and identified a PIGH
cDNA.\textsuperscript{20} PIG-H is a 188-amino-acid ER-associated protein that resides on the cytoplasmic side. We found that PIG-A and PIG-H formed a complex.\textsuperscript{18} Although the role of PIG-H has not been clarified, it is an essential subunit of GPI-GnT because PIGH-defective cells do not express GPI-APs.

3. PIGC gene. The Orlean group isolated mutant yeast clones defective in GPI-AP biosynthesis and identified the GPI2 gene involved in the first step.\textsuperscript{21} Inoue in our laboratory found that the mammalian homolog of GPI2 complemented the GPI-AP biosynthesis defect in class C mutant cells and termed it PIGC.\textsuperscript{22} PIG-C is a 297-amino-acid ER-membrane protein with multiple hydrophobic regions. Although the role of PIG-C has not yet been clarified either, it is also an essential subunit of GPI-GnT because PIGC-defective cells do not express GPI-APs.
4. PIGQ gene. The Orlean group identified the yeast GPI1 gene, which is also involved in the first step. Watanabe in our laboratory identified a human homolog of GPI1 and found that PIG-A, PIG-C, PIG-H, and PIG-Q (originally called hGPI1) formed a complex and that PIG-Q was required for stable expression of the other subunits. PIG-Q, which is a 581-amino-acid ER-membrane protein, is not an essential component of GPI-GnT because PIGQ-defective cells have some surface GPI-AP expression. Watanabe also showed that affinity-isolated complexes of epitope-tagged PIG-A had GPI-GnT activity in vitro.

5. PIGP gene. Watanabe found another protein in the isolated complex of GPI-GnT, cloned the corresponding cDNA based on the N-terminal sequence of the protein, and termed it PIGP. We subsequently found that murine mutant T-cell clone 2.10 was defective in the first step and that the PIGP cDNA complemented the defect. PIG-P is a 134-amino-acid ER-membrane protein with two transmembrane domains and is an essential subunit of GPI-GnT. PIG-P interacted with PIG-A and PIG-Q within the GPI-GnT complex.

Watanabe also found the DPM2 protein in the GPI-GnT complex. DPM2 is one of the three subunits of dolichol-phosphate mannose (Dol-P-Man) synthase. GPI-GnT is three-fold less active in the absence of DPM2, suggesting that GPI biosynthesis is co-regulated with Dol-P-Man synthesis.

6. PIGY gene. Murakami in our laboratory found yet another protein in the isolated GPI-GnT and cloned a PIGY cDNA based on its N-terminal sequence. PIG-Y is a 71-amino-acid ER-membrane protein with two transmembrane domains. Murakami found that human Burkitt lymphoma Daudi cell was defective in the first step and was a PIGY null mutant. Daudi cells expressed low levels of GPI-APs, indicating that PIG-Y is not essential for GPI-GnT activity. PIG-Y associated directly with PIG-A, suggesting that PIG-Y may regulate the catalytic action of PIG-A.

These studies together established that GPI-GnT mediates GlcNAc transfer to PI on the cytoplasmic side of the ER and that it is a complex of seven proteins, namely PIG-A (catalytic subunit), PIG-H, PIG-C, PIG-Q, PIG-P, PIG-Y, and DPM2.

7. PIGL gene. Stevens isolated Chinese hamster ovary (CHO) mutant cells defective in the second step in GPI biosynthesis, de-N-acetylation of GlcNac-PI generating GlcN-PI. We isolated similar CHO mutant cells and termed them class L GPI-defective mutant cells. Using class L CHO cells as recipients of a cDNA library generated with a polyoma-virus-based vector, Nakamura and Inoue cloned a PIGL cDNA that complemented the class L mutations. PIGL encodes a 252-amino-acid PIG-L
proteins that reside on the cytoplasmic side of the ER. Watanabe demonstrated that PIG-L and its yeast homolog had GlcNAc-PI de-N-acetylace activity in vitro.31

8. PIGW gene. GlcN-PI is subsequently acylated (usually palmitoylated or rarely myristoylated) at the 2-position of the inositol ring, generating GlcN-(acyl)PI. Murakami and Siripanyapinyo in our laboratory found human and CHO cells defective in the inositol-acylation, and by expression cloning, identified a PIGW cDNA that restored inositol-acylation in these mutant cells.32 Isolated PIG-W protein had palmitoyl-CoA-dependent GlcN-PI acylation activity in vitro, indicating that PIG-W is an inositol-acylase.33 PIG-W is a 504-amino-acid ER-membrane protein with multiple transmembrane domains. Sagane et al. analyzed the membrane topology of Gwt1p, a yeast homolog of PIG-W, and concluded based on the membrane topology of essential amino acids that inositol-acylation occurs in the lumen of the ER.34

Taken together with the membrane topology of GPI-GnT' and PIG-L indicating that the first two steps occur on the cytoplasmic side, it was suggested that flipping of GlcN-PI from the cytoplasmic side to the luminal side occurs in the third step of the pathway, and it is then inositol-acylated in the fourth step. The gene(s) involved in the flipping of GlcN-PI remain to be identified (Fig. 2, step 3).

Houjou et al. determined the structures of the PI moiety in GlcNAc-PI, GlcN-PI, and GlcN-(acyl)PI by mass-spectrometry and found that GlcNAc-PI and GlcN-PI have similar PI structures to cellular PI, i.e. they are mainly 1-stearoyl (C18:0)-2-arachidonoyl (C20:4) diacyl PI. In contrast, GlcN-(acyl)PI was a mixture of 1-alkyl-2-acyl and diacyl forms with the former being the major form, suggesting that the lipid moiety changes in GlcN-(acyl)PI (Fig. 2, step 5).35 Kanazawa in our laboratory found that generation of the 1-alkyl-2-acyl form of GlcN-(acyl)PI is dependent upon a pathway in the peroxisome that generates 1-alkyl-glycerone-phosphate from dihydroxyacetone-phosphate, and that the acyl chain compositions of GlcN-PI and the diacyl form of GlcN-(acyl)PI are different, suggesting that diacyl GlcN-(acyl)PI is generated from GlcN-PI by inositol-acylation in step 4 and then converted to a mixture of 1-alkyl-2-acyl and diacyl GlcN-(acyl)PI in step 5 of the pathway (Fig. 2).36 A possible mechanism may be that the diacyl glycerol (or phosphatidic acid) part is exchanged with 1-alkyl-2-acyl or diacyl glycerol (or phosphatidic acid) derived from a putative donor phospholipid. The putative donor phospholipid may contain 1-alkyl-2-acyl and diacyl forms like phosphatidylethanolamine. The gene(s) required for this lipid remodeling have not yet been clarified.

9. PIGM gene. In the sixth step of the pathway, Man1 is transferred from Dol-P-Man to GlcN-(acyl)PI, generating Mano1-4GlcN-(acyl)PI. Maeda in our laboratory found that a GPI-AP-deficient cell line derived from Burkitt lymphoma Ramos cells was defective in the transfer of Man1 and cloned a PIGM cDNA using that cell line by expression cloning.37 PIG-M is a 423-amino-acid ER-membrane protein with multiple transmembrane domains and has a functionally-important DXD motif in the first luminal domain.38 These characteristics of PIG-M are compatible with those of ER-resident, Dol-P-monomosaccharide-dependent glycosyltransferases, suggesting that PIG-M is GPI-mannosyltransferase I.37

10. PIGX gene. Ashida in our laboratory found a GPI-AP-deficient mutant CHO cell line with a defect in the transfer of Man1. However, that mutant was not complemented by the PIGM cDNA. By expression cloning, Ashida cloned a PIGX cDNA that restored the Man1-transfer and surface GPI-AP expression. PIG-X is a single-transmembrane ER protein of 252 amino acids, and is associated with PIG-M. The stability of PIG-M is dependent upon its association with PIG-X.37

11. PIGV gene. The seventh step of the pathway is transfer of Man2 from Dol-P-Man to GlcN-(acyl)PI, generating Mano1-4GlcN-(acyl)PI. Kang and Hong in our laboratory established mutant CHO cell lines defective in the transfer of Man2. Using one of the mutant clones as the recipient of a cDNA library, they cloned a PIGV cDNA that restored the defect after transfection.39 PIG-V is a 493-amino-acid ER-membrane protein with multiple transmembrane domains and has a functionally-important HD motif in the first luminal domain. These characteristics are compatible with those of ER-resident, Dol-P-monomosaccharide-dependent glycosyltransferases, suggesting that PIG-V is GPI-mannosyltransferase II.

12. PIGN gene. The Conzelmann group identified yeast GPI7, which is involved in the transfer of a side branch to Man2, contains three motifs conserved in families of phosphodiesterases, and has two close homologs.39 Hong in our laboratory identified one of the mammalian GPI7 homologs, termed PIGN, and demonstrated that PIGN is required for the transfer of an EtNP side
branch to Man1 by generating PIGN-knockout murine F9 cells.40) PIG-N, GPI-EtNP transferase I, is an ER-resident protein of 931 amino acids with multiple transmembrane domains. PIGN is not essential for later steps in GPI biosynthesis and PIGN-knockout cells expressed GPI-APs at mildly reduced levels.40)

13. PIGB gene. Takahashi in our laboratory cloned a PIGB cDNA by expression cloning using class B Thy-1-deficient murine T-lymphoma cells. The class B mutant cell line was defective in the transfer of Man3 and the PIGB cDNA restored the defect after transfection.41) PIG-B is a 554-amino-acid ER-membrane protein with multiple transmembrane domains. PIG-B, PIG-Z, and two α-mannosyltransferases, ALG9 and ALG12, in the N-glycosylation pathway form a protein family, supporting the idea that PIG-B is GPI-mannosyltransferase III.42)

14. PIGO gene. Hong characterized the second mammalian homolog of yeast GP17, termed PIGO, knocked it out in murine F9 cells, and found that PIGO is required for transfer to Man3 of EtNP that acts to link GPI to proteins.43) PIG-O is a 1,101-amino-acid ER protein with multiple transmembrane domains. PIG-B, PIG-Z, and two α-mannosyltransferases, ALG9 and ALG12, in the N-glycosylation pathway form a protein family, supporting the idea that PIG-B is GPI-mannosyltransferase III.42)

15. PIGF gene. Inoue in our laboratory cloned a PIGF cDNA by expression cloning using class F Thy-1-deficient murine T-lymphoma cells. The class F mutant cell line was defective in the transfer of EtNP to Man3.44) PIG-F is a 219-amino-acid ER-membrane protein with two transmembrane domains. PIG-O is a catalytic component of GPI-EtNP transference III.43)

16. PIGG gene. Shishioh in our laboratory identified a mammalian ortholog of GP17, now termed PIGG, and confirmed that PIGG is involved in the transfer of EtNP to Man2.45) Human PIG-G, a 983-amino-acid ER protein with multiple transmembrane domains like PIG-O, forms a complex with PIG-F, is stabilized by PIG-F, and competes with PIG-O for binding to PIG-F.45) PIG-G is a catalytic component of GPI-EtNP transference II.

17. PIGZ gene. The Taron group demonstrated that human SMP3, now termed PIGZ, is involved in the transfer of the fourth Man as a side branch to Man3, generating EtNP-6(Man1-2)Mano1-2(EtNP-6)Mano1-4(FEtNP-2)Mano1-6GlcNAc1-6(acyl-2)-myo-inositol-phospholipid.46) PIG-Z is a 579-amino-acid ER protein with multiple transmembrane domains and is GPI-mannosyltransferase IV. As described above, PIG-B, PIG-Z, ALG9, and ALG12 form an α-mannosyltransferase family.42)

18. GPAA1 gene. The Riezman group reported yeast GAAl (for GPI Anchor Attachment 1), the first component of GPI transamidase to be identified.47) Hiroi et al. reported the structural and functional human homolog of GAAl (now termed GPAA1). Human GPAA1 is a 621-amino-acid ER protein with multiple transmembrane domains.48) Ohishi in our laboratory indicated that GPI transamidase is a protein complex by demonstrating that GPAA1 forms a complex with GPIS (now termed PIG-K),49) which is a catalytic component of GPI transamidase. The Menon group reported that a large luminal domain which between first and second transmembrane domains of GPAA1 is involved in interaction with other components of GPI transamidase.50)

19. PIGK gene. The Conzelmann group identified yeast GPIS as a catalytic component of GPI transamidase.51) The Medof group identified a human homolog of Gpi8p, termed PIG-K.52) PIG-K and Gpi8p have a cysteine protease motif that is involved in cleavage of the GPI attachment signal peptide and the generation of a substrate-enzyme complex linked by a thioester bond (Fig. 3).51,52) PIG-K is a 395-amino-acid protein with one transmembrane domain near the C-terminus.52)

20. PIGS gene. Ohishi isolated the GPI transamidase complex and identified two more components.53) Based on the N-terminal sequence, he cloned PIGS. PIG-S is a 555-amino-acid protein with two transmembrane domains near the N- and C-termini. Although the function of PIG-S has not been clarified, it is essential for the enzyme activity because PIGS knockout F9 cells do not express GPI-APs. PIG-S is associated with PIG-T in the complex.

21. PIGT gene. Ohishi cloned the PIGT gene based on the N-terminal sequence of the other component, which he found in the isolated GPI transamidase.53) PIG-T is a 578-amino-acid protein with one transmembrane domain near the C-terminus. PIG-T is disulfide-linked to PIG-K.54) PIG-T is also essential for the enzyme activity because PIGT knockout F9 cells do not express GPI-APs. In the absence of PIG-T, the expression levels of GPAA1 and PIG-K were very low, whereas PIG-S was expressed normally, indicating that PIG-T is critical for the stability of GPAA1 and PIG-K.55)

22. PIGU gene. Hong in our laboratory established a new CHO mutant cell line defective in the step of GPI attachment and cloned the gene responsible, termed PIGU.55) PIG-U protein was
identified as the fifth component of the isolated GPI transamidase. PIG-U is a 435-amino-acid protein with multiple transmembrane domains. The other four GPI transamidase components formed a complex in the absence of PIG-U, suggesting that PIG-U is positioned at the periphery of the enzyme complex.

**PGAP (for Post GPI Attachment to Proteins)**

*genes involved in structural remodeling of GPI after its attachment to proteins*

23. **PGAP1 gene.** After attachment of GPI, the main structure of GPI-APs is Protein-

EtNP-6Man1-2(EtNP-6)Mano1-4(EtNP-2)Mano1-6GlcNAc1-6(acyl-2)myoinositol-phospho-diacylglycerol. The inositol-linked acyl chain is removed soon after GPI attachment in most types of cells with the exception of erythrocytes, in which the inositol-linked acyl chain remains and inositol-acylated GPI-APs are expressed on the cell surface.56) PI-specific phospholipase C (PI-PLC) of bacteria, such as *Bacillus thuringiensis*, cleaves GPI and releases proteins from the cell surface. Because PI-PLC requires a 2-position hydroxyl group for cleavage, inositol-acylated GPI-APs are resistant to PI-PLC. In search of an inositol-deacylase, Tanaka and Maeda in our laboratory established a CHO mutant cell line with GPI-APs resistant to PI-PLC and cloned the gene responsible, termed **PGAP1**.57) PGAP1, a 922-amino-acid ER-membrane protein, has a lipase motif in the luminal domain, indicating that PGAP1 is the inositol-deacylase itself (Fig. 4). In **PGAP1** mutant CHO cells, ER-to-Golgi transport of GPI-APs was three-fold slower than in wild-type cells, although the cell surface expression levels of GPI-APs were normal.57) Fujita found that the GPI-APs in **PGAP1**-defective cells did not bind to the cargo receptor for GPI-APs, a p24 complex consisting of p241, p242, and p2461, which is required for efficient packaging GPI-APs into COPII-coated transport vesicles (Fig. 4).58) It is possible that GPI-APs bearing the inositol-linked acyl chain are transported by bulk flow.

24. **PGAP5/MPPE1 gene.** To identify genes involved in the transport of GPI-APs, Maeda established a CHO cell system for monitoring ER-to-plasma membrane transport of GPI-APs that can be used to derive mutant CHO cells with slow transport of GPI-APs.59) Using this system, Fujita isolated a mutant clone, C19, in which ER-to-Golgi transport of GPI-APs was several-fold slower than in wild-type cells, and cloned the gene responsible, termed **PGAP5** (official gene symbol: **MPPE1**).60) PGAP5 is a 396-amino-acid, single-membrane-spanning protein with a luminal, dimetal-containing phosphoesterase domain. GPI-APs expressed on the surface of the **PGAP5**-deficient CHO cells had an EtNP side branch linked to Man2, which does not exist in GPI-APs derived from wild-type cells, indicating that C19 cells were defective in removal of the Man2-linked EtNP side branch and that PGAP5 is an enzyme that removes this EtNP (Fig. 4). Fujita further demonstrated that PGAP5 was localized at the ER exit sites and that GPI-AP recruitment into the ER exit sites was impaired in C19 cells, thereby accounting for the GPI-AP-specific delay in ER-to-Golgi transport.60)

25. **PGAP3 gene.** As described above (see section 8. **PIGW gene**), the structure of the PI moiety changes in GlcN-(acyl)PI from the diacyl form to a mixture of 1-alkyl-2-acyl as the major form and the diacyl form as a minor form. The latter profile is similar to that of the GPI-anchors of cell-surface GPI-APs, but there is a major difference in the sn2-linked fatty acids. GlcN-(acyl)PI contains various unsaturated chains, such as oleic, arachidonic, and docosatetraenoic acids, whereas GPI-APs usually have stearic acid. Based on the study of **PGAP2**-defective CHO cells described in the next section, we proposed that GPI fatty acid remodeling occurs, in which the sn2-linked fatty acid is exchanged from an unsaturated chain to a saturated chain, stearic acid, and that the lyso-GPI-AP found in **PGAP2**-defective cells is an intermediate in the fatty acid remodeling (Fig. 4).61) Maeda then hypothesized that there must be a gene, termed **PGAP3**, involved in the elimination of the unsaturated fatty acid and that, if **PGAP3** is mutated in the **PGAP2**-defective clone, the decreased GPI-AP expression might be restored because two fatty chains are maintained. Indeed, Maeda established a double-mutant CHO cell line expressing almost normal levels of GPI-APs from the **PGAP2**-defective clone and determined that a mammalian homolog of yeast **PERI**, which was reported to be involved in similar fatty acid remodeling in yeast, is **PGAP3**.62) **PGAP3** is a 320-amino-acid Golgi-resident protein with seven transmembrane domains. **PGAP3** belongs to a hydrolase superfamily and is most likely to be GPI-AP-specific phospholipase A2, although the enzyme activity has yet to be demonstrated in vitro.

26. **PGAP2 gene.** Tashima and Maeda established a CHO mutant cell line, termed clone C84, that synthesized GPI normally but showed greatly reduced surface expression of GPI-APs. They
clarified the mechanisms of the abnormality by demonstrating that GPI-APs in C84 mutant cells were converted to the lyso-GPI form by losing a fatty acid before exiting the trans-Golgi network and that, after transport to the cell surface, the lyso-GPI-APs were cleaved by a phospholipase D, resulting in secretion of soluble GPI-APs lacking a phosphatidic acid moiety and reduced cell surface levels of GPI-APs. They cloned the gene responsible, termed PGAP2, by sorting C84 cells that restored the normal levels of GPI-APs after transfection of a cDNA library. PGAP2 is a 254-amino-acid membrane protein mainly expressed in the Golgi. PGAP2 is involved in the second step of GPI-AP fatty acid remodeling, in that the lyso-GPI-AP intermediate is reacylated by stearic acid (Fig. 4). There is no significant sequence homology between PGAP2 and known acyltransferases, and the issue of whether PGAP2 is the acyltransferase itself or a regulatory protein remains to be determined.

GPI deficiencies caused by somatic and germline mutations in PIG and PGAP genes

Paroxysmal nocturnal hemoglobinuria (PNH). PNH is an acquired hematopoietic stem cell disorder characterized by clonal blood cells deficient in surface expression of GPI-APs that appear in all the hematopoietic cell lineages. A major clinical symptom is intravascular hemolysis of the affected erythrocytes by their own complement proteins, manifested as hemolytic attacks associated with infections and as chronic low-level hemolysis caused by continuous activation of the complement alternative pathway. We established affected B-lymphoblastoid cell lines from two patients with PNH and demonstrated that they were defective in the first step of the GPI biosynthetic pathway and belonged to the class A complementation group. Takeda in our laboratory demonstrated in 1993 that transfection of the PIGA cDNA restored GPI-AP expression on these PNH cells, that the affected B-lymphoblastoid cells and affected granulocytes, but not wild-type B-lymphoblastoid cells from the same patients, had PIGA mutations, and that the PIGA gene is X-linked. Based on these results, we concluded that the GPI-anchor deficiency in PNH is caused by single-hit somatic mutations in the PIGA gene that occur in hematopoietic stem cells.

Owing to X-chromosome inactivation, only one PIGA allele is active in female hematopoietic stem cells and somatic mutations in the active PIGA cause GPI deficiency. Therefore, the X-linkage of PIGA accounts for the equal sex ratio in patients with PNH.

Among the 26 genes involved in GPI biosynthesis, transfer, and remodeling, only PIGA is X-linked and all of the others are autosomal. Two mutations in the autosomal genes are required to generate GPI-deficient cells, but two somatic mutations in the same gene are highly unlikely to occur, thereby accounting for the fact that PIGA is responsible for GPI deficiency in most of the patients analyzed.

Inherited GPI deficiencies (IGDs). The PIGA somatic mutations causative of PNH are mainly null mutations causing complete GPI-AP deficiency. These mutations are compatible with life because only clonal hematopoietic cells are affected and non-hematopoietic cells are not affected. The situation for germline mutations is different and only hypomorphic mutations are compatible with live birth. Clinical symptoms of individuals with IGDs are wide range and various among individuals as described below. The major reason for the heterogeneity in symptoms would be different extents of functional loss caused by different hypomorphic mutations. Dependent upon the amount of GPI generated by the residual biosynthetic activity, the different numbers of GPI-AP species would be affected because GPI attachment signal peptides of different GPI-APs have different efficiencies in GPI transamidation. This should result in phenotypic variation.

1. PIGM deficiency. The Karadimitoris group and our group collaborated to clarify the molecular basis of partial GPI-AP deficiency in three affected individuals from two consanguineous families and found in 2006 that the same single-base substitution in the promoter region of the PIGM gene was responsible. The homozygous mutation disrupted an Sp1-binding site, and caused reduced PIGM mRNA levels and partial reduction of GPI-APs on the surface of granulocytes, B-lymphoblastoid cells, and skin fibroblasts. The affected individuals suffered from seizures and thrombosis in hepatic and/or portal veins.

Murakami demonstrated that the affected B-lymphoblastoid cells regained normal expression of GPI-APs after a 2-day culture in medium containing sodium butyrate that inhibited histone deacetylases and increased acetylation of the PIGM promoter and transcription, which in turn increased PIG-M expression and GPI biosynthesis. Based on these
in vitro data, the Karadimitoros group treated one of the affected individuals, who had severe seizures, with sodium butylate, resulting in increased levels of GPI-APs on blood cells and control of the seizures.\(^6^6\)

2. PIGV deficiency. Krawitz and colleagues identified mutations in PIGV by exome sequencing of DNA samples from three individuals with hyperphosphatasia with mental retardation syndrome (HPMRS, also termed Mabry syndrome).\(^6^7\) HPMRS is an autosomal recessive disorder characterized by intellectual disability and elevated levels of serum alkaline phosphatase (alkaline phosphatases are GPI-APs), often accompanied by seizures, facial dysmorphism, and various anomalies such as brachytelephalangy. Blood granulocytes from some of the individuals with PIGV mutations had partially reduced surface expression of CD16, a GPI-AP. Murakami collaborated with these researchers to determine the functional effects of the mutations on PIG-V function and demonstrated partial loss of functional activity using an assay in which a mutant PIGV cDNA was transfected into PIGV-defective CHO cells to determine the ability to restore the surface expression of GPI-APs by flow cytometry.\(^6^7\)

Murakami proposed a mechanism for the hyperphosphatasia based on an in vitro study with PIGV-defective CHO cells. In the ER of the PIGV-defective cells, the C-terminal GPI attachment signal peptide of a nascent protein, such as alkaline phosphatase, is cleaved by GPI transamidase and the major part of the protein is either secreted without GPI-anchoring or degraded by ER-associated degradation. The secretion accounts for the high serum levels of alkaline phosphatase.\(^6^6\)

3. PIGO deficiency. Krawitz and colleagues also identified mutations in PIGO by exome sequencing and/or Sanger sequencing of DNA samples from three individuals with HPMRS.\(^6^9\) Murakami collaborated with these researchers and demonstrated partial functional loss of PIG-O activity in restoring the surface GPI-AP expression in PIGO-defective CHO cells.\(^6^6\) Kuki and colleagues reported an individual with PIGO deficiency having HPMRS. CD16 and several other GPI-APs were significantly decreased on granulocytes as determined by flow cytometry, showing the usefulness of flow-cytometric determination of CD16 levels on blood granulocytes in the diagnosis of IGDs.\(^7^0\) HPMRS caused by PIGV and PIGO mutations are termed HPMRS type 1 and HPMRS type 2 (HPMRS1 and HPMRS2), respectively.

4. PGAP2 deficiency. Three research groups identified PGAP2 mutations in nine individuals with HPMRS and non-syndromic intellectual disability, mainly by exome sequencing.\(^7^1,7^2\) Murakami collaborated with these groups in determining the functional consequences of the mutations. As Tashima showed for PGAP2-defective CHO cells, when only the removal of the sn2-linked fatty acid occurred because of inefficient PGAP2-dependent reacylation during fatty acid remodeling, GPI-APs became lysoglycophosphatidyl-inositol (GPI)-APs, and were transported to the cell surface and secreted. Therefore, the mechanisms of secretion or hyperphosphatasia in PIGV/PIGO-defective cells and PGAP2-defective cells are different, comprising secretion before and after attachment of GPI, respectively. HPMRS caused by PGAP2 mutations is termed HPMRS type 3 (HPMRS3).

5. PIGN deficiency. Maydan and colleagues identified a homozygous PIGN mutation in seven individuals from a large consanguineous family who had multiple congenital anomalies with hypotonia and seizures. Six of the seven individuals died before 4 years of age.\(^7^3\) Ohba reported two affected siblings with compound heterozygous PIGN mutations with congenital anomalies, developmental delay, hypotonia, seizures, and progressive cerebellar atrophy. CD16 on blood granulocytes was reduced.\(^7^4\) PIGN deficiency is now termed MCAHS syndrome type 1 (for multiple congenital anomalies with hypotonia and seizures) or MCAHS1.

6. PIGA deficiency. Johnston and colleagues identified a PIGA mutation by X-chromosome exome sequencing in members of a family with an X-linked lethal disorder, including three deceased male children and two obligate carrier females. The affected children had cleft palate, neonatal seizures, contractures, central nervous system structural malformations, and other anomalies.\(^7^5\) More recently, different PIGA mutations were identified in three related males with neurodegeneration, cutaneous abnormalities, and systemic iron overload, and in a child with developmental delay, accelerated linear growth, facial dysmorphisms, mildly elevated serum alkaline phosphatase, and progressive central nervous system abnormalities, thus expanding the spectrum of phenotypes caused by PIGA mutations.\(^7^6,7^7\) Inherited PIGA deficiency is now termed MCAHS type 2 (MCAHS2).

7. PIGL deficiency. By exome sequencing, Ng and colleagues identified homozygous and compound heterozygous PIGL mutations in six affected individuals from five families with CHIME syndrome,
characterized by colobomas, heart defects, ichthyosisiform dermatosis, mental retardation, and ear anomalies. CD59 expression was reduced in fibroblasts and B-lymphoblastoid cells from some of the affected individuals. 78)

8. PIGT deficiency. Kvarnung and colleagues found a homozygous PIGT mutation in four individuals from a consanguineous family who had intellectual disability, hypotonia, seizures, and facial features in combination with abnormal skeletal, endocrine, and ophthalmologic findings. 79) Granulocyte CD16 was reduced. The affected individuals had hypophosphatasia, rather than hyperphosphatasia, 79) being consistent with our proposal that secretion of alkaline phosphatase is dependent upon GPI transamidase. 58) The hypophosphatasia accounts for the skeletal abnormalities seen in these individuals. PIGT deficiency is termed MCAHS type 3 (MCAHS3).

Perspectives

In the GPI biosynthetic pathway, the genetic bases of two steps are currently unknown. One is the step of GlcN-PI flipping. A mutant cell line defective in this step has never been obtained. As the screening of mutant cells defective in surface expression of GPI-APs is almost saturated, it is unlikely that the flipping depends on a single gene. It appears that either redundant genes are present or the putative gene involved in GlcN-PI flipping is also required for another function essential for cells, thereby preventing the isolation of mutant cells. Although a biochemical approach is an alternative, an assay for GlcN-PI flipping that is sufficiently sensitive to follow the purification of the flipase needs to be established for this approach to be feasible. The other is the step of lipid remodeling in GlcN-(acyl)PI. It is unlikely that this step is critical for later steps of the pathway because the genetic bases of various mutant cells defective in the addition of Man1 have been clarified. Again, a biochemical approach is an alternative and an assay for lipid remodeling needs to be developed.

To date, IGDs caused by hypomorphic mutations in seven PIG and PGAP genes have been reported. IGDs caused by mutations in several other PIG and PGAP genes are under study and will be reported soon. Loss-of-function mutations in any one of the 26 PIG and PGAP genes described in this review would cause IGDs. Owing to the highly active effort applied to exome sequencing, it may not take such a long time to identify GPI deficiencies caused by all of the PIG and PGAP genes. With the accumulation of more genetic, biochemical, cell biological, and clinical information from many more cases of IGDs, we will achieve better understanding of the mechanistic bases of various symptoms, improved ways for diagnosis, and hopefully useful treatment measures.

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Profile

Taroh Kinoshita was born in 1951 and started his research career in 1974 with studies on bactericidal insect proteins at the Graduate School of Agricultural Sciences at the University of Tokyo, after graduating from the Faculty of Agricultural Science of the same University. In PhD course in Osaka University Medical School, he studied mammalian complement system. He did 3-year post-doctoral research in the Department of Pathology, New York University School of Medicine from 1982. He demonstrated that blood cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) consist of normal cells and cells deficient in decay-accelerating factor (DAF), and that the latter population is highly sensitive to complement, showing the critical role of DAF in self-nonself discrimination in the complement system. From 1985 to 1990, as a Research Associate and then an Assistant Professor in Osaka University Medical School, he studied mouse complement receptors and demonstrated that complement receptor type 2 is important for antibody response. From 1990 to date, as a Professor in the Research Institute for Microbial Diseases, Osaka University, he has been studying biosynthesis of glycosylphosphatidylinositol (GPI)-anchor and molecular basis of PNH. In 1993, he for the first time cloned PIG-A that is required for GPI biosynthesis. Since then, he has cloned most of the genes involved in GPI pathway. He demonstrated that PIG-A mutation causes deficiency of GPI-anchor in PNH and that PIG-A mutation occurs somatically in hematopoietic stem cell, clarifying acquired nature of the disease and why only blood cells are affected. From 2003 to 2007, he was Director of the Research Institute for Microbial Diseases, Osaka University. From 2007, he has been a Deputy-Director of WPI Immunology Frontier Research Center, Osaka University.