

Chapter 2

Structural Changes of GPI Anchor After Its Attachment to Proteins: Functional Significance

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Introduction

One hundred and fifty or more of human proteins are post-translationally modified by a glycolipid, termed glycosylphosphatidylinositol (GPI) that anchors proteins to the outer leaflet of plasma membrane (Orlean and Menon 2007; Kinoshita et al. 2008). While GPI-anchored proteins (GPI-APs) have wide range of functions, such as hydrolytic enzymes, adhesion molecules, receptors and protease inhibitors, they share common membrane-anchors. GPI is synthesized in the endoplasmic reticulum (ER) from phosphatidylinositol (PI) via at least 11 steps (Fig. 2.1). Preamsembled GPI is transferred en bloc by GPI transamidase to the C-terminus of proteins having a C-terminal GPI-attachment signal peptide. A unique feature of GPI-anchor is that GPI structure is dynamically modified during transport of GPI-APs to the cell surface. Structural remodeling of lipid and glycan moieties in the ER is critical for efficient recruitment of GPI-APs into ER-exit site and association with their cargo receptors for transportation to the Golgi apparatus (Kinoshita et al. 2013). Fatty acid remodeling of GPI in the Golgi is important for homodimerization and raft association. In this chapter, these structure-biology relationships are described.

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A. Chakrabarti, A. Surolia (eds.), *Biochemical Roles of Eukaryotic Cell Surface Macromolecules*, Advances in Experimental Medicine and Biology 842,
DOI 10.1007/978-3-319-11280-0_2

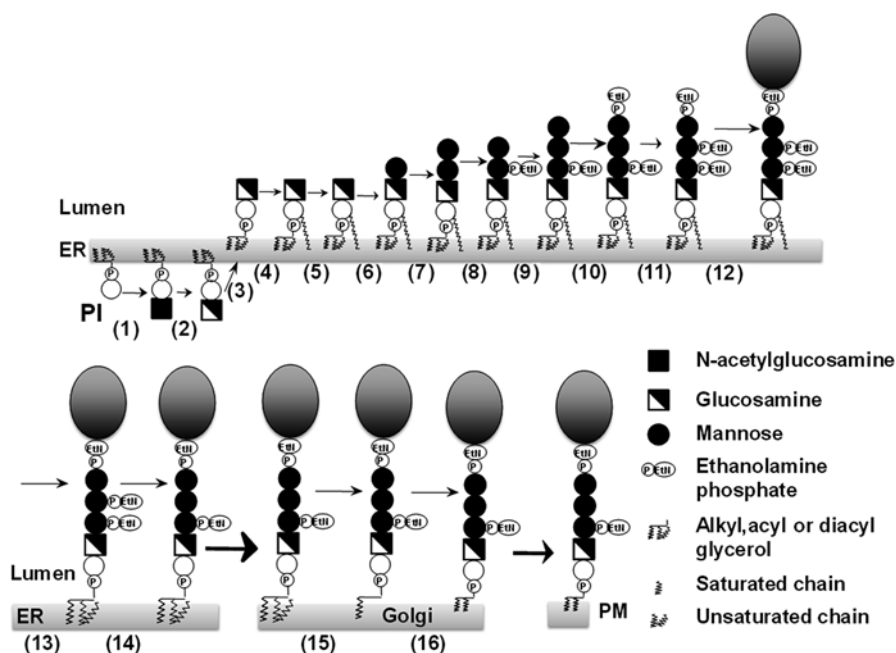


Fig. 2.1 Schematic representation of biosynthesis of GPI-APs in mammalian cells. GPI is synthesized from phosphatidylinositol (PI) in the ER (steps 1–11). Preassembled GPI is transferred to the protein's carboxyl-terminus (step 12). After two remodeling reactions in the ER (steps 13 and 14), GPI-AP is transported to the Golgi apparatus where GPI's sn2-linked fatty acid is remodeled (steps 15 and 16) before transport to the plasma membrane (PM)

Biosynthesis of GPI and Its Attachment to Proteins

GPI biosynthesis is initiated using cellular PI in the ER (Fig. 2.1). The first reaction is transfer of N-acetylglucosamine (GlcNAc) to PI from UDP-GlcNAc to generate first intermediate GlcNAc-PI (step 1). Step 1 is mediated by GPI-GlcNAc transferase (GPI-GnT) complex consisting of PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, PIG-Y and DPM2 proteins (Watanabe et al. 2000; Murakami et al. 2005). GlcNAc-PI is de-N-acetylated by deacetylase PIG-L to generate second intermediate glucosamine (GlcN)-PI (step 2) (Nakamura et al. 1997; Watanabe et al. 1999). These two reactions occur on the cytoplasmic side of the ER. GlcN-PI then flips into the luminal side with currently unknown mechanism (step 3). The inositol in GlcN-PI is acylated (mainly palmitoylated or myristoylated) to generate third intermediate GlcN-(acyl)PI (step 4). PIG-W, acyl-CoA-dependent acyltransferase, mediates step 4 (Murakami et al. 2003). The lipid moiety in GlcN-(acyl)PI is remodeled by currently unknown mechanism (step 5). See part 3 in the next section for lipid remodeling of GlcN-(acyl)PI. Two mannoses are transferred from dolichol-phosphate-mannose to generate Man α 1-6Man α 1-4GlcN α 1-6(acyl)PI by PIG-M/PIG-X complex (Maeda

et al. 2001; Ashida et al. 2005) and PIG-V (Kang et al. 2005), respectively (steps 6 and 7). Ethanolamine phosphate (EtNP) side branch is transferred by PIG-N from phosphatidylethanolamine to the first mannose generating $\text{Man}\alpha 1\text{-6(EtNP2)Man}\alpha 1\text{-4GlcN}\alpha 1\text{-6(acyl)PI}$ (step 8) (Hong et al. 1999). Third mannose is then transferred by PIG-B from dolichol-phosphate-mannose to generate $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6(EtNP2)Man}\alpha 1\text{-4GlcN}\alpha 1\text{-6(acyl)PI}$ (step 9) (Takahashi et al. 1996). Two EtNP are sequentially transferred from phosphatidylethanolamine to the third and second mannoses by PIGO/PIGF complex (Inoue et al. 1993; Hong et al. 2000) and PIGG/PIGF complex (Shishioh et al. 2005), respectively, to generate $\text{EtNP6Man}\alpha 1\text{-2(EtNP6)Man}\alpha 1\text{-6(EtNP2)Man}\alpha 1\text{-4GlcN}\alpha 1\text{-6(acyl)PI}$ (steps 10 and 11). The EtNP linked to the third mannose acts to bridge GPI to proteins.

The preassembled GPI is then transferred to the carboxyl terminus of proteins by GPI transamidase consisting of PIG-K, GPAA1, PIG-S, PIG-T and PIG-U (step 12) (Ohishi et al. 2000, 2001; Hong et al. 2003). Proteins that are to be GPI anchored have a GPI attachment signal peptide at their carboxyl terminus. The signal peptide is cleaved and replaced with the preassembled GPI. The amino group of the “bridging EtNP” makes an amide bond with the newly generated carboxyl terminus by transamidation (Orlean and Menon 2007).

GPI Remodeling Reactions That Occur After Attachment to Proteins

Inositol-Deacylation by PGAP1 in the ER

Shortly after attachment to proteins, the inositol-linked acyl chain is removed by PGAP1, inositol-deacylase, in the ER (step 13) (Tanaka et al. 2004). This lipid remodeling is necessary for efficient ER-to-Golgi transport of GPI-APs. Specifically, recognition of GPI-APs by the cargo receptor for recruitment into COPII-coated transport vesicles requires elimination of the acyl chain (Fujita et al. 2011). In PGAP1 defective Chinese hamster ovary (CHO) cells, ER-to-Golgi transport of DAF/CD55, a GPI-AP, is threefold slower than in wild-type cells due to inefficient binding to the cargo receptor (Tanaka et al. 2004).

Removal of an EtNP Side Branch from the Second Mannose by PGAP5 in the ER

In addition to inositol-deacylation, elimination of the EtNP from the second mannose is required for binding to the cargo receptor (step 14) (Fujita et al. 2011). In PGAP5 defective CHO cells, ER-to-plasma membrane transport of GPI-APs is fourfold slower (Fujita et al. 2009).

Fatty Acid Remodeling of GPI-AP in the Golgi

Cellular PI is exclusively diacyl form and its predominant species is 1-stearoyl(C18:0)-2-arachidonoyl(C20:4) PI. The structures of the PI moiety in GPI intermediates that were accumulated in mutant cells defective in each of the early biosynthetic steps, were determined by mass-spectrometry and were found that GlcNAc-PI and GlcN-PI have PI structures similar to cellular PI in both CHO cells and human lymphoma cells. In contrast, GlcN-(acyl)PI was a mixture of 1-alkyl-2-acyl and diacyl forms with the former being the major form. The major species of the former were 1-stearyl-2-oleoyl, -2-arachidonoyl, and -2-docosatetraenoyl PI. It was, therefore, suggested that the lipid moiety changes in GlcN-(acyl)PI (Houjou et al. 2007). It was also found 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 diacylGlcN-PI and then converted to a mixture of 1-alkyl-2-acyl and diacyl GlcN-(acyl)PI (step 5 in Fig. 2.1) (Kanzawa et al. 2009). A possible mechanism of the conversion may be that the diacyl glycerol part is exchanged with 1-alkyl-2-acyl or diacyl glycerol derived from a putative donor phospholipid. Alternatively, phosphatidic acid part is exchanged. The putative donor phospholipid may contain 1-alkyl-2-acyl and diacyl forms. It was pointed out that phosphatidylethanolamine has chain compositions similar to that of the remodeled GlcN-(acyl)PI and is a candidate of the donor lipid (Kanzawa et al. 2009). The gene(s) required for this lipid remodeling in the ER have not yet been clarified.

It was then 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. Mutant CHO cells defective in synthesis of 1-alkyl-glycerone-phosphate generated only the diacyl form of GlcN-(acyl)PI (Kanzawa et al. 2009). It is likely that 1-alkyl-glycerone-phosphate is converted to the putative donor lipid in the ER, which is then used in lipid remodeling reaction (step 5 in Fig. 2.1). Consistent with these findings, fibroblasts from patients with peroxisomal disorders, Zellweger syndrome and rhizomelic chondrodysplasia punctata, were defective in generation of 1-alkyl-2-acyl form of GPI anchors, suggesting that a lack of or a decrease in the 1-alkyl-2-acyl form of GPI anchors might be related to some of the symptoms of these patients (Kanzawa et al. 2012).

1-Alkyl-2-acyl PI is the major form of protein-bound GPI-anchors in mammalian cells and diacyl PI is a minor form. Therefore, the profile of lipid moiety of GlcN-(acyl)PI after lipid remodeling is similar to that of the GPI-anchors of cell-surface GPI-APs. However, there is a major difference in the sn2-linked fatty acids. 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 GlcN-(acyl)PI contains various unsaturated chains, such as oleic, arachidonic, and docosatetraenoic acids.

We established a CHO mutant cell line, termed clone C84 that synthesized GPI normally but showed greatly reduced surface expression of GPI-APs. We 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. Based on the study of C84 CHO cells, 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 C84 cells is an intermediate in the fatty acid remodeling (steps 15 and 16) (Tashima et al. 2006).

Tashima et al. cloned the gene responsible for C84 defect, 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 (step 16) (Tashima et al. 2006). 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.

Maeda et al. then hypothesized that there must be a gene, termed *PGAP3*, involved in the elimination of the unsaturated fatty acid (step 15) and that, if *PGAP3* is mutated in the *PGAP2*-defective cells, the decreased GPI-AP expression might be restored because two fatty chains are maintained. Indeed, Maeda et al. established a double-mutant CHO cell line expressing almost normal levels of GPI-APs from the *PGAP2*-defective C84 cells and determined that a mammalian homolog of yeast *PER1*, which was reported to be involved in similar fatty acid remodeling in yeast, is *PGAP3*. *PGAP3* is a 320-amino-acid Golgi-resident protein with seven trans-membrane 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. These findings together demonstrated that fatty acid remodeling of GPI-APs occurs in the Golgi of mammalian cells (Maeda et al. 2007).

Biological Significance of Fatty Acid Remodeling

Raft association and homodimerization of GPI-APs: Raft association is the prominent characteristic of GPI-APs (Schroeder et al. 1994). GPI-APs expressed on the surface of *PGAP3*- and *PGAP2*-double defective CHO cells were not efficiently recovered in the detergent resistant membrane fraction (Maeda et al. 2007). Similarly inefficient recovery of GPI-APs into the detergent resistant membrane fraction was seen in peritoneal macrophages, spleen T-lymphocytes and embryonic fibroblasts from *Pgap3*-knockout mice (Murakami et al. 2012a; Wang et al. 2013). This profile of GPI-APs lacking fatty acid remodeling is compatible with the idea that two saturated fatty chains are required for raft association of GPI-APs and hence the presence of unsaturated chain is inhibitory (Schroeder et al. 1994).

It was reported that GPI-APs form transient homodimers on the cell surface with a life-time of 200 ms and that the homodimers are the major state on unstimulated cells.

For homodimerization, both protein and GPI parts are important. Upon ligand binding, these minimal rafts of GPI-APs make clusters with signaling capability (Suzuki et al. 2012). We confirmed homodimerization of GPI-APs and demonstrated that the fatty acid remodeling is necessary for the dimerization (Seong et al. 2013).

PGAP2 and PGAP3 deficiencies: Hypomorphic mutations were found in *PGAP2* gene in nine individuals with hyperphosphatasia with mental retardation syndrome (HPMRS, also termed Mabry syndrome) and non-syndromic intellectual disability, mainly by whole-exome sequencing (Hansen et al. 2013; Krawitz et al. 2013). 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. In 2010, Krawitz and colleagues identified hypomorphic mutations in *PIGV* by whole-exome sequencing of DNA samples from three patients with HPMRS (Krawitz et al. 2010). Blood granulocytes from some of the patients with *PIGV* mutations had partially reduced surface expression of CD16, a GPI-AP. Using an assay in which mutant *PIGV* cDNAs were transfected into *PIGV*-defective CHO cells to determine the ability to restore the surface expression of GPI-APs by flow cytometry, the functional effects of the mutations on *PIG-V* function were found to cause a partial loss of functional activity. Murakami et al. proposed a mechanism for the hyperphosphatasia based on an in vitro study with *PIGV*-defective CHO cells (Murakami et al. 2012b). 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. Subsequently, hypomorphic mutations in *PIGO* (Krawitz et al. 2012) and *PIGW* (Chiyonobu et al. 2014) were found in individuals with HPMRS.

It is not known due to unavailability of cell samples from affected individuals whether the surface levels of GPI-APs on cells from individuals with *PGAP2* mutations are decreased. The hyperphosphatasia is an indication that GPI-APs are released from the cells. The mechanism of the hyperphosphatasia in *PGAP2*-deficiency must be different from that in *PIGV*-deficiency. Alkalinephosphatase released from *PIGV*-defective cells has never been modified by GPI whereas one released from *PGAP2*-defective cells is once GPI-anchored and after cell surface expression is released. As Tashima et al. showed for *PGAP2*-defective CHO cells, when only the removal of the sn2-linked fatty acid by *PGAP3* occurred because of inefficient *PGAP2*-dependent reacylation during fatty acid remodeling in the Golgi, GPI-APs became lyso-GPI-APs, and were transported to the cell surface and secreted (Tashima et al. 2006).

Four mutations in *PGAP3* were identified in five individuals with HPMRS (Howard et al. 2014). All four mutations caused severe reduction in cellular *PGAP3* function either by mislocalization of the mutant *PGAP3* proteins to the ER, a loss of activity, or non-sense mediated mRNA decay. In one of the individuals, reduction in the surface level of CD16 was confirmed. The exact mechanisms of reduction in the

cell surface levels of GPI-APs and release of GPI-APs (hyperphosphatase) in *PGAP3* deficiency are unclear at the moment. But GPI-APs bearing unremodeled fatty acids are not well associated with lipid rafts and may be released under some unknown conditions. Therefore, the mechanisms of secretion or hyperphosphatase in *PIGV*-defective cells, *PGAP3*-defective cells and *PGAP2*-defective cells are different.

These results from studies on *PGAP2*- and *PGAP3*-deficiencies indicate that proper fatty acid remodeling in the Golgi is critical for stable cell surface expression of GPI-APs. Impairment in the fatty acid remodeling causes abnormalities in neuronal functions, such as intellectual disability and seizures.

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Macromolecules

Chakrabarti, A.; Surolia, A. (Eds.)

2015, IX, 411 p. 127 illus., 72 illus. in color., Hardcover

ISBN: 978-3-319-11279-4