thematic review series

Thematic Review Series: Glycosylphosphatidylinositol (GPI) Anchors: Biochemistry and Cell Biology

Biosynthesis of GPI-anchored proteins: special emphasis on GPI lipid remodeling

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Abstract Glycosylphosphatidylinositols (GPIs) act as membrane anchors of many eukaryotic cell surface proteins. GPIs in various organisms have a common backbone consisting of ethanolamine phosphate (EtNP), three mannoses (Mans), one non-*N*-acetylated glucosamine, and inositol phospholipid, whose structure is EtNP-6Manα-2Manα-6Manα-4GlNα-6*myo*inositol-P-lipid. The lipid part is either phosphatidylinositol of diacyl or 1-alkyl-2-acyl form, or inositol phosphoceramide. GPIs are attached to proteins via an amide bond between the C-terminal carboxyl group and an amino group of EtNP. Fatty chains of inositol phospholipids are inserted into the outer leaflet of the plasma membrane. More than 150 different human proteins are GPI anchored, whose functions include enzymes, adhesion molecules, receptors, protease inhibitors, transcytotic transporters, and complement regulators. GPI modification imparts proteins with unique characteristics, such as association with membrane microdomains or rafts, transient homodimerization, release from the membrane by cleavage in the GPI moiety, and apical sorting in polarized cells. GPI anchoring is essential for mammalian embryogenesis, development, neurogenesis, fertilization, and immune system. Mutations in genes involved in remodeling of the GPI lipid moiety cause human diseases characterized by neurological abnormalities. Yeast *Saccharomyces cerevisiae* has >60 GPI-anchored proteins (GPI-APs). GPI is essential for growth of yeast. In this review, we discuss biosynthesis of GPI-APs in mammalian cells and yeast with emphasis on the lipid moiety.—Kinoshita, T., and M. Fujita. **Biosynthesis of GPI-anchored proteins: special emphasis on GPI lipid remodeling. *J. Lipid Res.* 2016.** 57: **6–24.**

Supplementary key words glycosylphosphatidylinositol • fatty acid remodeling • peroxisome • genetic disorder

Glycosylphosphatidylinositols (GPIs) are glycolipids that are ubiquitously found in eukaryotic organisms (1). GPIs act as membrane anchors of many cell surface proteins after they are covalently attached to the C termini of the proteins as a posttranslational modification. GPIs are also found as free glycolipids (2, 3). GPIs in various organisms have a common backbone consisting of ethanolamine phosphate (EtNP), three mannoses (Mans), one non-*N*-acetylated glucosamine (GlcN), and inositol phospholipid, whose structure is EtNP-6Manα-2Manα-6Manα-4GlNα-6*myo*inositol-P-lipid (**Fig. 1A**) (4–6). The lipid part is either phosphatidylinositol (PI) of diacyl or 1-alkyl-2-acyl form, or inositol phosphoceramide (6–8). GPIs are attached to the proteins via an amide bond between the C-terminal carboxyl group and an amino group of EtN. Fatty chains of inositol phospholipids are inserted into the outer leaflet of the plasma membrane, while proteins are not directly inserted into the membrane (Fig. 1A). GPI anchor is the only form of posttranslational modification by glycolipid.

More than 150 different human proteins are GPI anchored (UniProt) (9). Functions of these GPI-anchored

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Abbreviations: *Ace*, angiotensin converting enzyme; AGPS, alkylglycerone phosphate synthase; ALP, alkaline phosphatase; BIQ, 1-[4-butylbenzyl]isoquinoline; *bkr*, beaker; *BST*, bypass of Sec13; CHO, Chinese hamster ovary; DHAP-AT, dihydroxyacetone phosphate acyltransferase; Dol-P-Man, dolichol-phosphate-mannose; ER, endoplasmic reticulum; ERES, ER exit site; EtNP, ethanolamine phosphate; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcN, non-*N*-acetylated glucosamine; GlcNAc, *N*-acetylglucosamine; GPI, glycosylphosphatidylinositol; GPI-AP, glycosylphosphatidylinositol-anchored protein; HHAT, hedgehog acyltransferase; HPMRS, hyperphosphatasia with mental retardation syndrome; IPC, inositol-phospho-ceramide; Man, mannose; MBOAT, membrane bound *O*-acyl transferase; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D; RCDP, rhizomelic chondrodysplasia punctate; Ser, serine; Sia, sialic acid; SRP, signal recognition particle; TMD, transmembrane domain.

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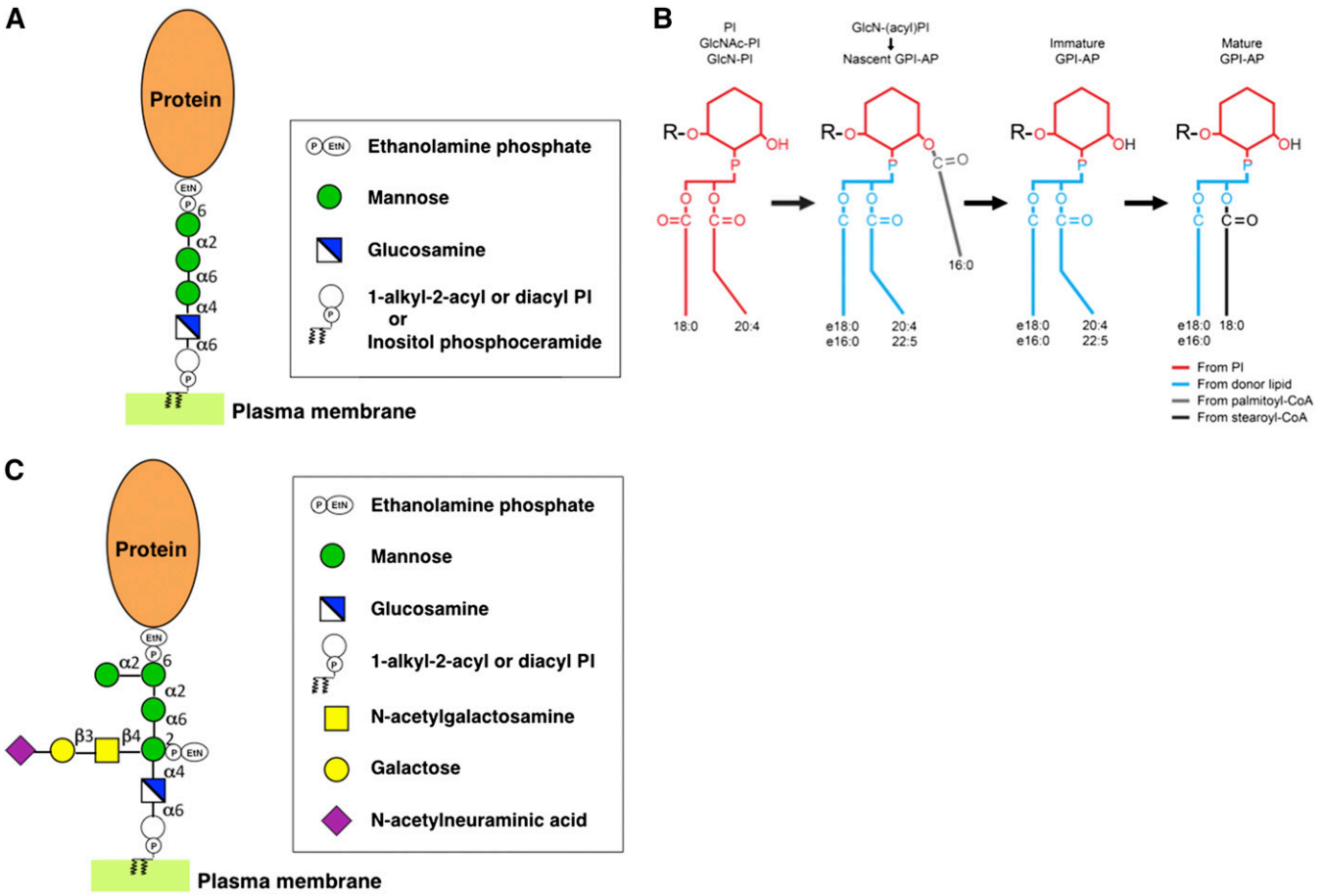

Fig. 1. A: Common backbone of GPI anchors. The common backbone of GPI anchors, consisting of EtNP, three Mans, GlcN, and inositol phospholipid, is conserved in wide varieties of eukaryotes. The entire protein is anchored to the outer leaflet of the plasma membrane by fatty chains of inositol phospholipid. B: Dynamic changes of the PI moiety along biosynthesis of GPI-APs. Most of the mammalian cellular PI and the first two GPI intermediates are diacyl forms bearing *sn*2-linked unsaturated fatty acid. In contrast, the major forms of mature GPI-APs on the cell surface have 1-alkyl-2-acyl PI bearing *sn*2-linked saturated fatty acid (usually stearic acid). This structural difference occurs during GPI-AP biosynthesis through two types of remodeling reactions, diacyl to diradyl remodeling in the ER and fatty acid remodeling in the Golgi. C: Mammalian GPI-APs. The first Man in the common core is ubiquitously modified by EtNP at the 2-position. In some proteins, the fourth Man is attached to the third Man. Yet in other proteins, the first Man is modified by the β4GalNAc side branch, which may be elongated by β3Gal and Sia. Modified from Figure 1 in (197) with permission.

proteins (GPI-APs) are various, including >40 enzymes [alkaline phosphatases (ALPs), 5′-nucleotidase, dipeptidase, and others], several adhesion molecules (contactins, glypicans, CD48, and others), receptors (folate receptors, GDNF receptor alphas, FcγRIIIb, and others), protease inhibitors (RECK), transcytotic transporters (GPIHBP1), and complement regulatory proteins (CD55 and CD59). Because of GPI modification, mammalian GPI-APs have unique characteristics, such as association with membrane microdomains or membrane rafts enriched in sphingolipids and cholesterol (10), transient homodimerization (11), release from the membrane by cleavage in the GPI moiety (12–15), and apical sorting in polarized cells (16). GPI anchoring of proteins is essential for mammalian embryogenesis, development, neurogenesis, fertilization, and immune system (12, 14, 17–21). Yeast *Saccharomyces cerevisiae* has >60 GPI-APs. GPI anchors are essential for growth of yeast (22). In this review, we discuss biosynthesis of GPI-AP in mammalian cells and yeast with emphasis on the lipid moiety.

STRUCTURAL CHARACTERISTICS OF MAMMALIAN GPI AND GPI-AP

The lipid moiety of mammalian GPI-APs has two unique characteristics compared with cellular free PI, from which GPI is generated. First, a major form of GPI-APs is the 1-alkyl-2-acyl form and diacyl PI is a minor form, whereas free PI is mostly the diacyl form and contains only a trace amount, if any, of the 1-alkyl-2-acyl form (Fig. 1B) (8, 23, 24). Second, the *sn*2-linked fatty acid in GPI-APs is mostly stearic acid, a saturated 18 carbon chain (C18:0), whereas free PI has mainly arachidonic acid, a polyunsaturated chain (C20:4) (Fig. 1B) (23, 24). The latter characteristic fatty chain composition of GPI, i.e., both *sn*1- and *sn*2-linked fatty chains are saturated in a vast majority of GPI-APs, is critically related to two unique properties of GPI-APs, i.e., association with membrane microdomains and transient homodimerization (25, 26). These two characteristic lipid structures in GPI are results of two lipid remodeling reactions (25, 27) (see below).

The non-*N*-acetylated GlcN is a unique characteristic of the glycan part of GPIs. GlcNs in other glycoconjugates are mostly *N*-acetylated, or some GlcNs in glycosaminoglycans are *N*-sulfated. In contrast, *N*-acetylglucosamine (GlcNAc), attached to PI in the first step in biosynthesis, is de-*N*-acetylated in the next step. Because of this non-*N*-acetylated GlcN, inositol phospholipid of GPI can be released from GPI and GPI-AP by deamination by nitrous acid (1, 28).

The common backbone is variously modified in different organisms and in different cell types in one species. In mammalian cells, the 2-position of the first Man (Man1) linked to GlcN in the backbone is ubiquitously modified by an EtNP side branch (Fig. 1C) (1, 8). In some mammalian proteins, such as rat Thy-1, the fourth Man (Man4) is attached to the third Man (Man3) via α1,2 linkage (Fig. 1C) (4). Yet in other proteins, such as human and porcine dipeptidases, β-*N*-acetylgalactosamine (GalNAc) is attached to 4-position in Man1 (8). This side branch GalNAc can be elongated by β1,3-galactose (Gal) and sialic acid (Sia) in some proteins, such as prion protein (Fig. 1C) (29).

BIOSYNTHESIS OF MAMMALIAN GPI AND ATTACHMENT TO PROTEINS

GPI is synthesized in the endoplasmic reticulum (ER) and is en bloc transferred to proteins by GPI transamidase. Biosynthesis of GPI is initiated on the cytoplasmic face of the ER by transfer of GlcNAc from UDP-GlcNAc to PI, generating the first intermediate GlcNAc-PI (step 1 in **Fig. 2**). This reaction is mediated by GPI-GlcNAc transferase, the most complex monoglycosyltransferase, consisting of seven proteins, PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, PIG-Y, and DPM2 (30–32). GlcNAc-PI is de-*N*-acetylated to GlcN-PI by a deacetylase, PIG-L (step 2 in Fig. 2) (33, 34). GlcN-PI is next translocated to the luminal face by an unknown mechanism (step 3 in Fig. 2). It is thought to be mediated by a "flippase," which has not been identified (35). Many of genes involved in GPI biosynthesis were cloned by expression cloning, taking advantage of mutant Chinese hamster ovary (CHO) cells and other cells defective in each one of the biosynthetic steps (36). However, cells defective in the "flipping" step have never been established, although the mutant screen seems to be already saturated (37–40). It seems that either a putative flippase additionally has a role essential for cell survival or the flip is mediated by redundant enzymes.

GlcN-PI that is flipped to the luminal face is then acylated by acyltransferase PIG-W at the 2-position of the inositol ring, generating GlcN-(acyl)PI (step 4 in Fig. 2) (41, 42). GlcN-(acyl)PI is subjected to lipid remodeling that converts the diacyl PI moiety to a mixture of 1-alkyl-2-acyl PI, the major form, and diacyl PI (step 5 in Fig. 2) (43). The exact remodeling reaction and the enzyme that catalyzes the reaction are yet to be clarified (see below).

The Man1 is transferred to the remodeled GlcN-(acyl) PI from dolichol-phosphate-Man (Dol-P-Man), generating Man-GlcN-(acyl)PI (step 6 in Fig. 2) (44). A complex of PIG-M, a catalytic component, and PIG-X, a stabilizing component, is GPI mannosyltransferase 1 that catalyzes this reaction (45, 46). The second Man (Man2) is transferred from Dol-P-Man by PIG-V, GPI mannosyltransferase 2, generating Man-Man-GlcN-(acyl)PI (step 7 in Fig. 2) (47). A side-branch EtNP is attached to the 2-position of

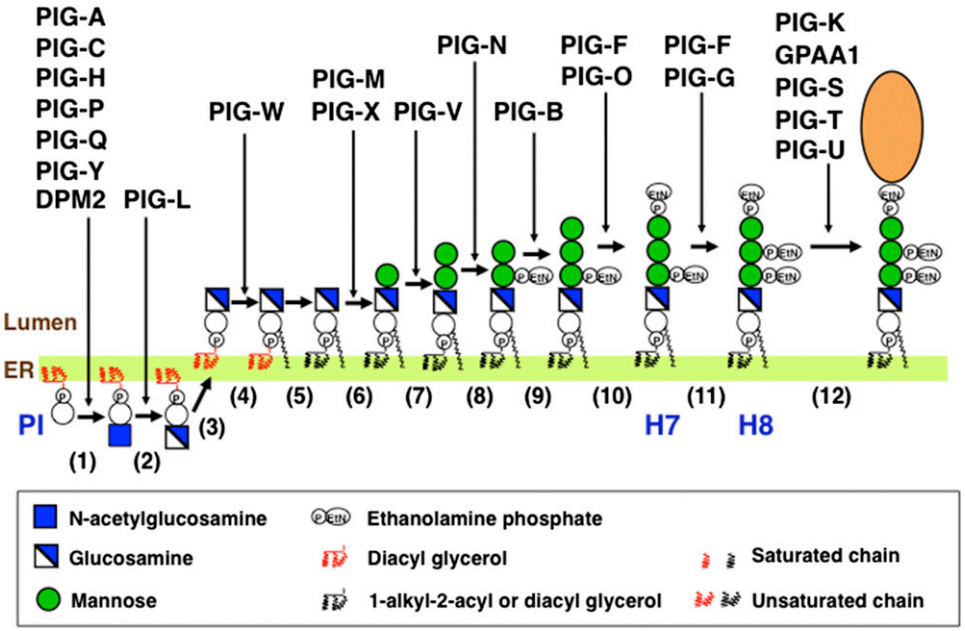

Fig. 2. Biosynthesis and protein-attachment of GPI in the ER of mammalian cells. The complete precursor of GPI, termed H8, is synthesized from PI by 11 stepwise reactions and en bloc transferred to proteins. More than 20 PIG genes, shown above the biosynthetic pathway, are involved. Modified from Figure 2 in (197) with permission.

Man1 to generate Man-(EtNP)Man-GlcN-(acyl)PI (step 8 in Fig. 2). The donor of EtNP is PE and PIG-N is GPI-EtNP transferase 1 that catalyzes this step (48). The Man3 is transferred from Dol-P-Man by PIG-B, GPI mannosyltransferase 3, generating Man-Man-(EtNP)Man-GlcN-(acyl)PI (step 9 in Fig. 2) (49). The so-called "bridging EtNP" that links GPI to proteins is attached to the 6-position of Man3 from PE by GPI-EtNP transferase 2 consisting of PIG-O, a catalytic component, and PIG-F, a stabilizing component (step 10 in Fig. 2) (50–52). The product, EtNP-Man-Man-(EtNP)Man-GlcN-(acyl)PI, is termed H7 and is competent for attachment to proteins (53). H7 is usually further modified by a side-branch EtNP attached to the 6-position of Man2 to generate EtNP-Man-(EtNP)Man-(EtNP)Man-GlcN-(acyl)PI, termed H8 (53). This reaction is mediated by GPI-EtNP transferase 3 consisting of PIG-G, a catalytic component, and PIG-F, a stabilizing component (step 11 in Fig. 2) (54). H8 is the "mature" precursor usually used to modify proteins. In some GPI-APs, Man4 is attached. Although the exact step at which Man4 is transferred has not been determined in the mammalian system, Man4 in yeast is transferred before attachment of the bridging EtNP (55). Man4 transfer is mediated by PIG-Z, GPI mannosyltransferase 4 (56).

H8 and H7 are posttranslationally attached to a protein's C terminus by transamidation (step 12 in Fig. 2) (57, 58). Preproproteins of GPI-APs have N-terminal signal sequence for ER translocation and C-terminal signal sequence for GPI attachment (**Fig. 3**) (57). The C-terminal GPI attachment signal sequence consists of four consecutive elements from the N-terminal side of the ω site (to which GPI is attached) to the C terminus: *1*) an unstructured linker of about 10 amino acids from site ω-11 to ω-1; *2*) ω and ω+2 sites with short side chains; *3*) five to ten hydrophilic amino acids; and *4*) 15–20 amino acid hydrophobic stretch (59). Serine (Ser), Asn, Asp, Gly, Ala, and Cys are known to act as ω site amino acids, and substitution of ω with another amino acid, such as proline, impairs GPI attachment (60). Software that predicts GPI attachment and ω sites is available online (61). Preproproteins are translocated into the ER lumen via translocon. A recent study with yeast demonstrated that the majority of its GPI-APs are translocated posttranslationally, while the rest are cotranslationally translocated in a signal recognition particle (SRP)-dependent manner (62). A more recent study reported evidence that the majority of human GPI-APs use SRP-dependent translocation; whereas, prion protein uses a SRP-independent posttranslational translocation path involving Sec62/63 (38). The preproproteins translocated into the ER lumen undergo N-terminal signal peptide removal and attachment to GPI. It is thought that they are transiently inserted into the ER membrane with the C-terminal hydrophobic stretch until attachment to GPI (Fig. 3).

GPI transamidase that catalyzes GPI attachment consists of five proteins, PIG-K, GPAA1, PIG-S, PIG-T, and PIG-U (63–67). PIG-K, a caspase-like cysteine protease family protein, attacks the peptide bond between the ω and ω+1 amino acids, cleaves off the C-terminal GPI attachment signal peptide, and generates substrate-enzyme intermediate, in which ω site amino acid is linked to the catalytic cysteine via a thioester bond (Fig. 3) (68). GPAA1 presents an amino group in bridging EtNP of the mature GPI precursor to nucleophilic attack of the thioester, resulting in attachment of GPI's EtN to the ω amino acid via an amide bond (69). PIG-T is linked to PIG-K via a disulfide bond and probably stabilizes the enzyme complex (70). PIG-S and PIG-U are also essential for GPI transamidase, although their exact roles are unclear.

MATURATION OF MAMMALIAN GPI-AP DURING TRANSPORT TO THE CELL SURFACE

Nascent GPI-APs formed by GPI transamidase are still immature and undergo at least three remodeling reactions to become mature GPI-APs. These remodeling reactions occur en route to the cell surface. The first reaction occurring in the ER is inositol deacylation by PGAP1, an inositol-deacylase (step 13 in **Fig. 4**) (see below for details) (71). Then the EtNP side branch linked to Man2 is removed by PGAP5, an EtNP phosphodiesterase (step 14 in Fig. 4) (72). PGAP1 is widely distributed in the ER; whereas, PGAP5 is restricted to the ER exit sites (ERESs) (71, 72). After these two remodeling reactions, GPI-APs are associated with a cargo receptor for packaging into COPII-coated transport vesicles in the ERESs. Because

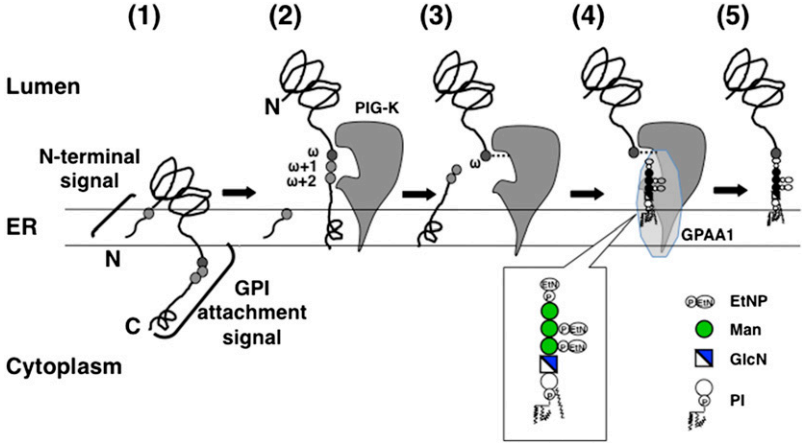

Fig. 3. Attachment of GPI to proteins by GPI transamidase. Preproprotein has an N-terminal signal for ER translocation and a C-terminal signal for GPI attachment (step 1). After translocation into the ER lumen, the N-terminal signal is removed and the C-terminal signal is recognized by PIG-K (step 2). PIG-K cleaves a peptide bond between ω and ω+1 amino acids, generating a substrate-enzyme complex linked via a thioester bond (step 3). The thioester-linked intermediate is attacked by GPI presented by GPAA1 (step 4) completing transamidation (step 5). For simplicity, PIG-S, PIG-T, and PIG-U are not depicted. Reproduced with permission from (198).

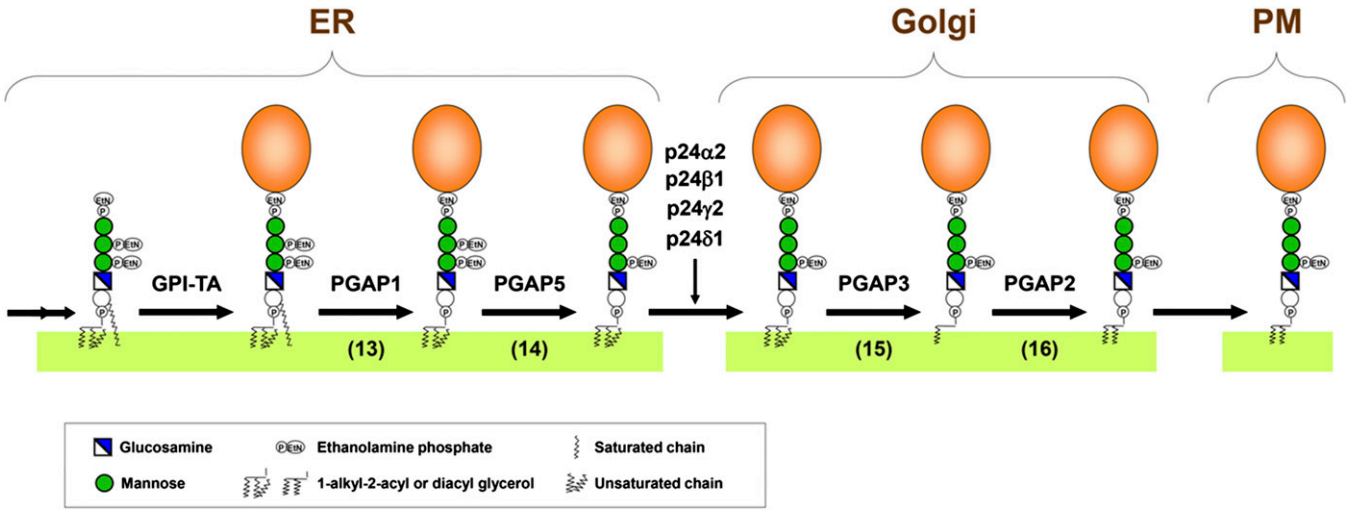

Fig. 4. Maturation of mammalian GPI-AP during ER-to-plasma membrane (PM) transport. Nascent GPI-APs formed by GPI transamidase (GPI-TA) undergo two reactions, inositol-deacylation (step 13) and removal of the EtNP side branch from Man2 (step 14) in the ER. A cargo receptor consisting of four p24 proteins is involved in ER-to-Golgi transport. In the Golgi, GPI-APs undergo fatty acid remodeling (steps 15 and 16), generating mature GPI-APs. Modified from Figure 4 in (197) with permission.

GPI-APs are inserted only to the luminal leaflet of the ER membrane that lacks direct access to the cytoplasmic COPII coat proteins, transmembrane cargo receptors are required for coupling GPI-APs to COPII coat proteins. Both inositol-deacylation and removal of EtNP from Man2 are required for binding to the cargo receptor (73). Therefore, only the properly remodeled GPI-APs are recognized by the cargo receptor. It is conceivable that these remodeling reactions are coordinated with the protein folding process so that properly folded and remodeled GPI-APs are incorporated into transport vesicles. How such coordination is regulated is to be clarified.

The cargo receptor for GPI-APs is a complex of four p24 proteins, TMED9 (also known as p24α2), TMED2 (p24β1), TMED5 (p24γ2), and TMED10 (p24δ1) (73–75). All p24 proteins are type 1 transmembrane proteins consisting of an N-terminal Golgi dynamic domain, an α-helical coiled-coil domain, a transmembrane domain (TMD), and a short C-terminal cytoplasmic domain (76). Members of the p24 family of proteins are divided into four subfamilies, α, β, γ, and δ (77). Evidence suggests that complexes are formed by proteins, one each from the four subfamilies, although it is unclear how complexes are assembled (78). Subfamilies β and δ have only one member, TMED2 (p24β1) and TMED10 (p24δ1), respectively (77), and they are essential for complex formation. Thus, when either of them is lost by gene knockdown or knockout, proteins in the three other subfamilies become unstable (79). The human α subfamily has two members, TMED9 (p24α2) and TMED4 (p24α3) (human p24α1 is a pseudogene), and TMED9 (p24α2), but not TMED4 (p24α3), seems to be involved in GPI-AP transport. The γ subfamily has five members and likely has a determining cargo recognition repertoire. TMED5 (p24γ2), but not the four other p24γ proteins, is involved in GPI-AP transport (79). Domain swapping between TMED5 (p24γ2) and TMED1 (p24γ1) and site-directed mutagenesis revealed that the α-helical coiled-coil domain, especially its juxta-membrane region of TMED5 (p24γ2), is critical for association with GPI-AP; whereas, the Golgi dynamic domain, TMD, and the cytoplasmic domain are interchangeable (79). The juxta-membrane region of TMED5 (p24γ2) might interact with the glycan part of GPI. Whether the lipid part of GPI is recognized by p24 cargo receptor is currently unclear.

The short cytoplasmic tails of p24 proteins contain motifs recognized by Sec24 proteins of the COPII coat. It was shown by RNAi knockdown experiments that of four Sec24 isoforms, Sec24C and Sec24D are necessary for efficient transport of GPI-APs, suggesting that Sec24C and -D associate with p24 cargo receptor complexes (75).

In vitro data suggest that GPI-APs may dissociate from the p24 cargo receptor complex in the ERGIC/Golgi lumen due to acidic pH (73). In the Golgi, GPI-APs are subjected to fatty acid remodeling in that unsaturated fatty acid at the *sn*2 position is replaced by saturated fatty acid, usually stearic acid. The removal of unsaturated fatty acid is mediated by a Golgi resident membrane protein, PGAP3, that is a GPI-specific phospholipase A2 (step 15 in Fig. 4) (25). Another Golgi-resident membrane protein, PGAP2, is required for reacylation of the lysoGPI-APs (step 16 in Fig. 4) (see below for details) (80).

The addition of a GalNAc-containing side chain to some of the GPI-APs also occurs most likely in the Golgi. Three glycosyltransferases, β4GalNAc transferase, β3Gal transferase, and a sialyltransferase, are to be identified, and molecular mechanisms of protein-selective and cell-type-specific side-chain modification are to be clarified (8).

The properly fatty acid-remodeled GPI-APs become associated with membrane microdomains or rafts, transient liquid-ordered nanoclusters of membrane constituents, and are transported to the cell surface (25). Molecular mechanisms of GPI-AP transport from the trans-Golgi network to the plasma membrane have not been clarified. Upon clustering by multivalent ligands of GPI-APs or

antibody against GPI-APs, some GPI-APs transduce signals for proliferation or motility. Two long saturated fatty chains of GPI-APs facilitate trans-bilayer interactions with long acyl-chain-containing phosphatidylserine. Such lipid-to-lipid interaction is necessary for trans-bilayer coupling of GPI-APs in the outer leaflet to the cytoplasmic signal transduction machinery and actin filaments (81).

In polarized cells, such as epithelial cells, GPI-APs are usually transported to the apical surface with some exceptional GPI-APs, such as prion protein, which is transported to the basolateral surface (16). Mechanisms of apical sorting of GPI-APs have been intensively studied (10). Oligomerization of GPI-APs in the trans-Golgi network seems to be critical for selective transport to the apical surface [see detailed discussion in a recent review (82, 83)].

DYNAMIC CHANGES OF THE PI MOIETY ALONG BIOSYNTHESIS FROM PI TO GPI-AP

It is remarkable that the structure of the PI moiety dynamically changes during biosynthesis of GPI-APs (Fig. 1B). Five steps (steps 4, 5, 13, 14, and 15) in the biosynthetic pathway, as described below, are related to structural changes in the PI moiety (**Table 1**). PI in mature mammalian GPI-APs is divided into three parts according to their origins: *1*) only inositol or the inositol phosphate part is derived from original cellular PI; *2*) 1-alkyl glycerol or 1-alkyl glycerol phosphate is from a donor lipid for lipid remodeling that occurs in the ER; and *3*) the *sn*2-linked fatty acid, usually stearic acid, is derived from the acyl-CoA used in fatty acid remodeling that occurs in the Golgi.

Inositol acylation

The 2-position of inositol in GlcN-PI is acylated in biosynthetic step 3, generating GlcN-(acyl)PI (41). The acyl chain is mostly palmitate, while myristate is found in a small fraction (84). The inositol-linked acyl chain is maintained until GPI is attached to proteins and is removed soon after protein attachment before exit from the ER (85). Therefore, it is a transient modification of PI usually lacking in GPI-APs. In mutant cells defective in inositol-acylation of GlcN-PI, transfers of three Mans and the EtNP side-branch to Man2 occur, although less efficiently than in normal cells; however, transfer of the bridging EtNP does not occur (42). Therefore, inositol-linked acyl chain is required for the action of GPI EtNP transferase 2.

The inositol-acylation is mediated by PIG-W in mammalian cells and by Gwt1p in yeast (42, 86). Isolated PIG-W generates GlcN-(acyl)PI from GlcN-PI and palmitoyl-CoA in vitro. Therefore, PIG-W is an acyltransferase itself (42). PIG-W is an ER membrane protein with 12 TMDs and has no apparent sequence homology to known acyltransferases. It was shown by determining the orientation of conserved residues in Gwt1p and PIG-W that the inositol-acylation occurs in the luminal side of the ER (87). PIG-W thus represents a new family of acyltransferases that acylates glycolipid on the luminal side of the ER.

Orientation of conserved amino acids in Gwt1p/PIG-W indicates that palmitoyl-CoA is used for acylation on the luminal side of the ER. Other examples of acyl-CoA-dependent acylation in the lumen of the ER/Golgi include palmitoylation of Ser in Wnt proteins, palmitoylation of hedgehog proteins, and octanoylation of ghrelin (88–90). These reactions are mediated by membrane bound *O*-acyl transferase (MBOAT) family acyltransferases bearing seven to ten TMDs (91–93). It is thought that acyl-CoA molecules generated on the cytoplasmic side of the ER/Golgi membrane are translocated into the luminal side by these MBOAT proteins and are used in acylation reactions (94). A possible model could be similar to a mechanism envisaged for *N*-acetylation of GlcN residue during degradation of heparan sulfate in the lysosome (95). For the *N*-acetylation, the acetyl group is first transferred from the cytoplasmic acetyl-CoA to the catalytic histidine of an enzyme acetyl-CoA:α-glucosaminide *N*-acetyltransferase, and then is used in the lysosome lumen to acetylate an amine of GlcN (95). Acetyl-CoA:α-glucosaminide *N*-acetyltransferase has 11 TMDs. PIG-W, although it does not belong to the MBOAT family, has 12 TMDs. It is conceivable that PIG-W also has an ability to translocate palmitoyl-CoA into the ER lumen for inositol-acylation.

Diacyl to alkyl-acyl lipid remodeling

The majority of the PI moiety in GPI-APs isolated from mammalian cells is the 1-alkyl-2-acyl form, and the diacyl form is less common. Almost all free PI in mammalian cells is the diacyl form, suggesting that the fatty chain composition of GPI changes during biosynthesis. By LC-ESI-MS/MS, Houjou et al. (84) analyzed fatty chain compositions of free PI, and the first three GPI intermediates, GlcNAc-PI, GlcN-PI, and GlcN-(acyl)PI accumulated in the *PIGL*-, *PIGW*-, and *DPM2*-defective CHO cells, respectively, to

TABLE 1. Defects in GPI biosynthesis/remodeling involving lipid moiety

Step	Reaction	Gene	Organelle	Biochemical Defect	Biochemical Phenotype	Disorder	Manifestation
4	Inositol acylation	*PIGW*	ER	GPI synthesis defect	Decreased GPI-AP levels	HPMRS5	DD/ID, Sz, HP
5	PI remodeling	Not identified	ER	Defective alkyl-acyl GPI synthesis	Only diacyl GPI-AP generated	Unknown	Unknown
13	Inositol deacylation	*PGAP1*	ER	Inositol-acylation remained	GPI-AP bearing 3 fatty chains	IGD	DD/ID, encephalopathy, hypotonia, CVI
15	Fatty acid remodeling	*PGAP3*	Golgi	Unsaturated acyl chain remained	Defective raft association	HPMRS4	DD/ID, Sz, HP, hypotonia
16	Fatty acid remodeling	*PGAP2*	Golgi	Defect in reacylation of lysoGPI-AP	Generation of lysoGPI-AP	HPMRS3	DD/ID, Sz, HP, hypotonia, organ anomalies

Steps correspond to those in Figs. 2 and 4. DD/ID, developmental delay/intellectual disability; CVI, cerebral visual impairment; HP, hyperphosphatasia; IGD, inherited GPI deficiency; Sz, seizures.

determine when the fatty chain composition changes. The vast majority of free PI in CHO cells was diacyl PI having 18:0 and 20:4 chains, as expected from previous reports on mammalian PI (23, 24). GlcNAc-PI and GlcN-PI maintained similar chain compositions, the 18:0-20:4 diacyl form being predominant. In contrast, GlcN-(acyl)PI had a clearly different chain composition, i.e., nearly 60% was the 1-alkyl-2-acyl form and less than 40% was the diacyl form. The appearance of the 1-alkyl-2-acyl form in GlcN-(acyl)PI was similarly seen in a *DPM1*-defective mouse T-lymphoma cell line. Therefore, there is a lipid remodeling in GlcN-(acyl)PI in which more than half of the predominant diacyl glycerol moiety is replaced by 1-alkyl-2-acyl glycerol (84).

Generation of 1-alkyl-2-acyl GlcN-(acyl)PI is dependent upon the peroxisomal alkyl phospholipid biosynthetic pathway (43). Mutant CHO cells defective in either dihydroxyacetone phosphate acyltransferase (DHAP-AT), the first enzyme in the pathway (96), or alkylglycerone phosphate synthase (AGPS), the second enzyme (97), generated only diacyl GlcN-(acyl)PI and the diacyl form of GPI protein anchors (43). The 1-alkylglycerone-3-phosphate generated in the peroxisome is subsequently converted to 1-alkyl phospholipids, such as 1-alkyl-2-acyl PE and phosphatidylcholine, and ethanolamine- and choline-plasmalogens, in the ER (98). Some of these 1-alkyl phospholipids might be used as a donor substrate in diacyl to 1-alkyl-2-acyl remodeling of GlcN-(acyl)PI.

The composition of fatty acids in diacyl GlcN-(acyl)PI in DHAP-AT-defective CHO cells was different from that in free PI and GlcN-PI in that the *sn*2 chains contained not only arachidonic acid, but also various unsaturated fatty acids, such as oleic and docosapentaenoic acids. Similarly, diacyl species of GlcN-(acyl)PI in *DPM2*-defective alkyl phospholipid synthesis intact CHO cells contained various fatty acids other than arachidonic acid. These results indicate that there is diacyl to diacyl remodeling in GlcN-(acyl)PI. Taken together, fatty chain composition changes from the 1-stearoyl-2-arachidonoyl dominant diacyl form to a mixture of the 1-alkyl-2-acyl and diacyl forms in GlcN-(acyl)PI is a distinct step in the GPI biosynthetic pathway (43).

A possible reaction of this lipid remodeling is exchange of diacyl glycerol or the phosphatidyl moiety with a corresponding diradyl structure between GlcN-(acyl)PI and a donor lipid. In these cases, a putative lipid remodelase might have properties of phospholipase C or D. The donor substrate might be PE because most, if not all, species of 1-alkyl-2-acyl and diacyl glycerol of GlcN-(acyl)PI are found in PE, but not in phosphatidylcholine (43). These issues need to be clarified in future studies.

Deacylation of inositol from nascent GPI-APs

Nascent GPI-APs, immediately after attachment of GPI to proteins, have the inositol-linked acyl chain and undergo inositol-deacylation before their exit from the ER for transport to the Golgi apparatus (85). The deacylation is mediated by an ER-resident deacylase, PGAP1, which has nine TMDs and a typical lipase motif containing a catalytic Ser (71).

The inositol-linked acyl chain confers resistance to bacterial PI-specific phospholipase C (PI-PLC) and the deacylation restores PI-PLC sensitivity. PI-PLC uses a hydroxyl group at the 2-position of the inositol ring during hydrolysis of a phosphoester bond, and the product bears an inositol 1,2-cyclic phosphate at the terminus. When the 2-position is acylated, PI-PLC does not cleave GPI. An epitope including inositol 1,2-cyclic phosphate and GlcN is recognized by anti-CRD (cross-reacting determinant) antibody, which is common among various GPI-APs (99, 100).

An erythrocyte is an exceptional cell type in which the inositol-linked acyl chain is not removed in the ER, and GPI-APs on the erythrocyte surface have three fatty chains and are resistant to PI-PLC (101). It is thought that PGAP1 expression is downregulated with erythrocytic differentiation. The extra fatty chain may contribute to a stable expression on the surface of this long-living cell by preventing spontaneous release (the half-life of human erythrocytes in the blood is 60 days). It is also known that some GPI-APs, such as CD52 on human spleen cells and sperm (102) and ALP on certain cell lines (103), are resistant to PI-PLC and maintain an inositol-linked acyl chain. Therefore, inositol-deacylation is not always complete on nucleated cells.

In *PGAP1*-defective nucleated cells, GPI-APs are resistant to PI-PLC like those on erythrocytes and, in addition, ER-to-Golgi transport of GPI-APs is slow, taking three times more time (71). GPI-APs bearing the inositol-linked acyl chain are not associated with the p24 cargo receptors, accounting for slow ER-to-Golgi transport in *PGAP1*-defective cells (73).

Fatty acid remodeling of mammalian GPI-APs in the Golgi

Mature GPI-APs on various mammalian cells usually have stearic acid at the *sn*2 position and, therefore, two saturated fatty chains, because *sn*1 chains in phospholipids are usually saturated. A minor fraction of them have an unsaturated chain with one unsaturated bond. This is in contrast to chain compositions of GlcN-(acyl)PI in the ER, which have various unsaturated fatty acids at the *sn*2 position. GPI fatty acid remodeling achieves the fatty chain profile of mature GPI-APs. Two Golgi-resident membrane proteins are involved in fatty acid remodeling (25, 80). PGAP3 is required for removal of *sn*2-linked unsaturated chain and generation of lyso form intermediate. PGAP3 has seven TMDs and belongs to a membrane-bound hydrolase superfamily termed CREST (for alkaline ceramidase, PAQR receptor, Per1, SID-1, and TMEM8) (104). Although direct demonstration of the enzyme activity has not succeeded, it is almost certain that PGAP3 is a GPI-specific phospholipase A2. PGAP2, bearing five TMDs, is involved in reacylation of the lyso form intermediate. Stearic acid is preferentially used to reacylate the *sn*2 position. It is not clear whether PGAP2 is acyltransferase itself, because it does not have amino acid sequence homology to known acyltransferases. In yeast, an MBOAT acyltransferase, Gup1p, is involved in a similar fatty acid remodeling of GPI-APs that occurs in the ER (see below). The closest sequence homologs of mammalian MBOAT are hedgehog

acyltransferase (HHAT) and HHATL (94); however, HHAT and HHATL did not seem to be involved in GPI remodeling (T. Kinoshita, unpublished observation). There is evidence for substrate channeling during fatty acid remodeling because when PGAP3 HA-tagged at the C terminus was expressed in cells, lyso form GPI-APs were generated and secreted from the cell surface (105). Perhaps, a remodelase complex consisting of PGAP3 and PGAP2 carries out deacylation and reacylation steps progressively without releasing lyso form intermediate.

In *PGAP3*-defective cells, GPI-APs do not undergo fatty acid remodeling in the Golgi and are transported to and expressed on the cell surface (25). The cell surface levels of GPI-APs are only mildly affected. Those GPI-APs have *sn*2-linked unsaturated fatty acid (oleic, arachidonic, docosapentaenoic, and docosahexaenoic acids). The major *sn*1 chain in GPI-APs is the C18:0 alkyl chain. These fatty chain compositions are very similar to those in GlcN-(acyl) PI, indicating that the lipid structure does not change between GlcN-(acyl)PI and immature GPI-APs (84).

The fatty acid unremodeled GPI-APs are not recovered in detergent-resistant membrane after cells are lysed in cold 1% Triton X-100 (25). The reason for this is that an unsaturated fatty acid in GPI interferes with association of GPI-APs with liquid-ordered membrane. Therefore, it is interpreted that fatty acid unremodeled GPI-APs have reduced interaction with glycosphingolipids and cholesterol, and are not associated with membrane microdomains.

The situation in *PGAP2*-defective cells is quite different from that in *PGAP3*-defective cells, i.e., the cell surface levels of GPI-APs are severely affected and they are only less than 10% of the wild-type levels (80). In the absence of PGAP2 activity, reacylation does not occur and the intermediate lysoGPI-APs, bearing only one fatty chain, are transported to the cell surface and quickly secreted. The secreted GPI-APs in the culture medium lose their lipid moiety by the action of phospholipase D (PLD) (80). GPI-specific PLD present in serum and also expressed in various cells (106, 107) is thought to be responsible for this cleavage. GPI-specific PLD may cleave GPI in membrane-bound lysoGPI-APs to enhance release, or may cleave spontaneously released lysoGPI-APs in the medium.

DEFICIENCIES OF LIPID/FATTY ACID REMODELING AND INOSITOL ACYLATION AND DEACYLATION

Peroxisomal disorders and GPI anchor abnormality

Diacyl to 1-alkyl-2-acyl lipid remodeling in the ER requires a lipid donor containing 1-alkyl glycerol, which is yet to be identified. Generation of this putative donor lipid is dependent upon a peroxisomal pathway for 1-alkyl glycerone phosphate, as described above (43). Defects in genes for the first two reactions, DHAP-AT and AGPS, cause the peroxisomal disorders rhizomelic chondrodysplasia punctate (RCDP) types 2 and 3, respectively (108). AGPS has type 2 peroxisome targeting signal and is transported into the peroxisome by a PEX7-dependent mechanism. Defects in PEX7 cause RCDP type 1 (108). Loss-of-function mutations in PEX genes, such as PEX5, PEX16, and PEX19, involved in transport of a major group of peroxisome matrix proteins and membrane proteins, cause a more severe form of peroxisome disorder, Zellweger syndrome (109). RCDP and Zellweger syndrome are characterized by multiple clinical phenotypes, such as psychomotor defects, intellectual disability, seizures, and skeletal abnormalities (110). Fibroblasts from patients with RCDP types 1–3 and Zellweger syndrome are defective in generation of plasmalogens due to defective synthesis of 1-alkyl glycerone phosphate in the peroxisome. In addition, those fibroblasts are defective in generation of the 1-alkyl-2-acyl form of GPI and express only the diacyl form of GPI-APs (Table 1) (27). It is possible that a lack of 1-alkyl-2-acyl GPI is causally related to some of these symptoms.

Loss-of-function mutations in *PIGW*, *PGAP2*, and *PGAP3* cause HPMRS/Mabry syndrome by three different mechanisms

HPMRS/Mabry syndrome by PIGW mutations. Biallelic mutations in the *PIGW* gene were found in a Japanese boy with early-onset epilepsy initially diagnosed as West syndrome (111). He was born to nonconsanguineous healthy parents and showed profound developmental delay and constantly elevated serum ALP levels, as well as seizures. The cell surface levels of two GPI-APs, CD16 and CD24, on blood granulocytes were decreased by 90 and 60%, respectively. He was therefore diagnosed as having hyperphosphatasia with mental retardation syndrome (HPMRS), also termed Mabry syndrome (Table 1). HPMRS caused by *PIGW* mutations is termed HPMRS5 to differentiate it from those caused by mutations in *PIGV* (HPMRS1) (112, 113), *PIGO* (HPMRS2) (114), *PGAP2* (HPMRS3) (115, 116), and *PGAP3* (HPMRS4) (117) (http://www.ncbi.nlm.nih.gov/omim/?term=HPMRS).

Two identified mutations in *PIGW*, p.Thr71Pro and p.Met167Val, substituted amino acids that are conserved in mammalian PIG-W and yeast Gwt1p. Functional activity of mutant PIG-Ws can be measured by determining their ability to restore cell surface expression of GPI-APs after transfection into *PIGW*-defective CHO cells (42). Both mutant PIG-Ws had greatly decreased activity in this assay (111). It was apparent that these loss-of-function *PIGW* mutations caused decreases in the cell surface levels of GPI-APs and affected proper functions of neuronal cells and other cells.

Hyperphosphatasia in HPMRS1, -2, and -5 is dependent upon GPI transamidase. In cells defective in *PIGV*, *PIGO*, or *PIGW*, GPI biosynthesis stops in the middle of the pathway and incomplete GPIs bearing one or more Mans accumulate. Under such conditions, GPI transamidase acts on preproproteins of ALP and other GPI-APs, and generates substrate protein-enzyme intermediates linked by a thioester bond. In the absence of H8 or H7, these intermediates are eventually hydrolyzed, and soluble proteins lacking the GPI attachment signal peptide are then secreted. Cleavage between ω and ω+1 amino acids was

demonstrated in a model experiment using *PIGO*-defective CHO cells (118). Therefore, in these cells the GPI anchor is never attached and ALP becomes soluble after attack by GPI transamidase. In cells defective in early steps in the GPI biosynthetic pathway, GPI transamidase action to preproproteins is less efficient and preproproteins are degraded by ER-associated degradation. Indeed, hyperphosphatasia does not occur, or occurs only mildly, in *PIGA*-, *PIGQ*-, and *PIGL*-deficiencies (119–121). Balance between degradation and secretion due to cleavage by GPI transamidase seems to be affected by other unknown factors, such as genetic backgrounds, because highly elevated hyperphosphatasia associated with *PIGL*-deficiency was recently reported (122). Consistent with the GPI-transamidase-dependent mechanism of hyperphosphatasia, hypophosphatasia, rather than hyperphosphatasia, occurs in individuals with deficiency in *PIGT*, a component of GPI transamidase (123).

HPMRS/Mabry syndrome by PGAP2 mutations. There are two reports of nine individuals with *PGAP2* mutations from three consanguineous (Pakistani, Syrian, and Turkish) families and one nonconsanguineous (Finnish) family (115, 116). The affected individuals had intellectual disability, seizures, and hyperphosphatasia. Severely affected individuals also had hypotonia, brachytelephalangy, anorectal abnormality, aganglionic megacolon/Hirschsprung disease, heart defect, hearing impairment, and/or cleft palate. Information about cell surface levels of GPI-APs on blood cells, such as granulocyte CD16, was not available. In one of those families, CD55 and CD59 levels on lymphoblastoid cells were not affected. *PGAP2* deficiency, therefore, causes HPMRS/Mabry syndrome and has been termed HPMRS3 (Table 1).

Collectively, five missense mutations, p.Arg16Trp, p.Tyr99Cys, p.Leu127Ser, p.Thr160Ile, and p.Arg177Pro, were identified in four families. Functional activity of mutant *PGAP2* cDNAs bearing these mutations were assessed using *PGAP2*-defective CHO cells on which GPI-AP expression levels were less than 10% of parental cells (80). Transfection of wild-type *PGAP2* cDNA fully restored the parental levels of GPI-AP expression; whereas, restorations by the mutant *PGAP2* cDNAs were partial, indicating that they were hypomorphic mutations.

Hyperphosphatasia in HPMRS3 and -4 occurs due to defective fatty acid remodeling after GPI anchor attachment, but by different mechanisms. In HPMRS3, the PGAP2 defect causes termination of fatty acid exchange reactions after elimination of *sn*2-linked fatty acid (step 15 in Fig. 4), generating lysoGPI-APs, which are transported to the cell surface, secreted, and found in the medium as soluble proteins lacking lipid moiety (80).

HPMRS/Mabry syndrome by PGAP3 mutations. Loss-of-function mutations in *PGAP3* were found in five individuals from three families (117). Three of five were from a Pakistani consanguineous family. All five affected individuals had developmental delay, intellectual disability, and hyperphosphatasia. In one of them, mild reduction in the surface levels of CD16 and CD59 on blood granulocytes was shown. Therefore, *PGAP3* deficiency causes HPMRS/Mabry syndrome and has been termed HPMRS4 (Table 1).

Three missense mutations and one frame-shift mutation were identified. Functional activity of mutant *PGAP3* cDNA can be determined by using CHO cells defective in both *PGAP2* and *PGAP3* genes. The double-defective CHO cells express GPI-APs at almost normal levels (25). When *PGAP3* cDNA is transfected, the double-defective cells become like *PGAP2*-defective cells and the cell surface levels of GPI-APs decrease to lower than 10% of normal levels because of secretion. If mutations cause loss-of-function, a reduction in the surface GPI-AP levels is small. Among three missense mutations, p.Gly92Asp caused almost complete loss-of-function, whereas p.Pro105Arg and p.Asp305Gly caused only partial reduction in GPI-AP levels, indicating hypomorphic nature.

As described above, defects in PGAP3 cause surface expression of GPI-APs bearing unsaturated fatty acid at the *sn*2-position. These GPI-APs are not associated with membrane microdomains (25). Release of ALP, however, has not been demonstrated in vitro and the surface expression levels of GPI-APs on cultured cell lines are not decreased. It is likely that ALP release occurs in vivo under certain conditions. In fact, surface levels of various GPI-APs on lymphocytes, granulocytes, and peritoneal macrophages from *Pgap3*-knockout mice were significantly decreased, and CD16 and CD59 were decreased on granulocytes from one individual with *PGAP3* deficiency (117, 124). Perhaps, ALP and other GPI-APs, which are not associated with membrane microdomains, are more easily released from the cell surface by a cleavage of GPI or some other mechanisms.

Deficiency in PGAP1

PGAP1 mutations have been found in five families. A homozygous mutation in *PGAP1*, p.Leu197del, was found in two siblings from a consanguineous Syrian family. Both of the affected individuals had intellectual disability and encephalopathy (125). Biallelic *PGAP1* mutations, p.Pro92del and Lys308Asnfs*25, were found in a boy with cerebral visual impairment and intellectual disability (126). Another set of biallelic *PGAP1* mutations, p.Gln466* and p.Tyr524*, were found in a boy with cerebral visual impairment, hypotonia, delayed motor development, and encephalopathy (127). Siblings with encephalopathy, hypotonia, microcephaly, and retinal dystrophy, from a consanguineous Turkish family were shown to have an intronic homozygous splice variant, c.1090-2A>G (128). Yet another homozygous splice variant, c.1952+1G>T, was identified in siblings having spastic paraplegia, developmental delay, and encephalopathy (Table 1) (129).

Defects in inositol-deacylase cause cell surface expression of GPI-APs having acyl chain linked to inositol. ER-to-Golgi transport of GPI-APs is significantly affected in *PGAP1*-defective cells, as described above, although the steady state levels of cell surface GPI-APs are not affected (71). The abnormal GPI-APs on the cell surface are resistant to PI-PLC (71). Indeed, GPI-APs on lymphoblastoid cells

derived from the affected individuals with homozygous p.Leu197del and compound heterozygous p.Pro92del and Lys308Asnfs*25 were completely resistant to PI-PLC-mediated cleavage. Inositol-deacylase activity of mutant PGAP1 can be measured by determining PI-PLC sensitivity of GPI-APs after transfection into *PGAP1*-defective CHO cells. *PGAP1* cDNAs having each of these three mutations did not restore PI-PLC sensitivity, indicating that they are null mutations.

Heterozygous family members of the affected homozygous individuals are healthy carriers. It was demonstrated that GPI-APs on lymphoblastoid cells from the heterozygous family members are partially resistant to PI-PLC (125). Therefore, inositol-deacylation activity in normal cells is not in excess and heterozygous null *PGAP1* mutation causes haploinsufficiency. There may be some biological meaning in this limited level of PGAP1 activity in cells, which should be clarified by further investigation.

These studies with individuals with PGAP1 deficiency demonstrated that PGAP1 nullizygosity is compatible with life, but mainly affects functions of neuronal cells, causing clinical phenotypes such as intellectual disability, developmental delay, encephalopathy, cerebral visual impairment, and hypotonia, but rarely seizures. It is likely that slowed transport of GPI-APs from the ER might be related to neuronal abnormalities seen in individuals with *PGAP1* mutations. Other possible harmful effects of unremoved inositol-linked acyl chain might be that interactions with putative GPI-binding proteins are affected and that inositol-acylated GPI-APs are resistant to shedding by some GPI-cleaving enzyme. These possibilities need to be investigated.

Comparison with Pgap1-knockout and Pgap1-mutant mice

Pgap1-knockout mice were generated by homologous recombination in the C57BL/6 strain (130). Most homozygous mice had abnormal head development and died in the perinatal period. The abnormal phenotypes fit with otocephaly, which is characterized by agnathia (absence of the lower jaw). A study with a naturally occurring mutant mouse strain with forebrain abnormality, termed *oto* for otocephaly, revealed the mutated gene was *Pgap1* (131). Yet another mutant mouse strain generated by chemical mutagenesis showing holoprosencephaly, a forebrain abnormality, was termed beaker (*bkr*) and was shown to have a mutation in *Pgap1* (18). These phenotypes of *Pgap1*-defective mice are apparently much stronger than those of human individuals with *PGAP1*-null mutations who do not have gross abnormality in the forebrain. It was reported that holoprosencephaly was seen in C56BL/6 $Pgap1^{bkr}$, whereas 129S1 $Pgap1^{bkr}$ mice had normal morphology, indicating that forebrain phenotype is dependent upon genetic backgrounds (18). This is perhaps relevant to a lack of morphological forebrain abnormality in human individuals with *PGAP1* mutations.

Male *Pgap1*-knockout mice were infertile (130). Sperm from *Pgap1*-knockout mice did not migrate efficiently from uterus to oviduct after mating and they did not adhere to zona pellucida of oocytes in vitro. These phenotypes are shared with several other mutant mice with male infertility. In particular, sperm from angiotensin converting enzyme (*Ace*)-knockout and germ cell-specific GPI-AP (*Tex101*)-knockout mice have similar phenotypes (12, 15). ACE is a dual-specificity enzyme having a carboxy-dipeptidase activity necessary for converting angiotensinogen to angiotensin, and a GPI-cleaving activity independent from the carboxy-dipeptidase activity (12). Ace is involved in disappearance of Tex101 from sperm during maturation, most likely through its GPI-cleaving activity, and the Tex101 disappearance is necessary for sperm to gain fertility (15). It is tempting to speculate that Tex101 on *Pgap1*-knockout sperm, presumably bearing inositol-linked acyl chain, is resistant to Ace-mediated cleavage/disappearance. Whether GPI-cleaving activity of Ace against Tex101 is causally related to infertility of *Pgap1*-knockout sperm needs to be investigated.

UNIQUE STRUCTURES IN YEAST GPI ANCHORS

The basic structure of GPI in *S. cerevisiae* is similar to that found in mammals and other species, while its side-chain structure and lipid moiety are unique to yeast. In addition to the core structure, yeast GPI contains two additional Mans (**Fig. 5**). One Man (Man4) is transferred from Dol-P-Man to Man3 via an α1,2 linkage during GPI biosynthesis in the ER by the GPI mannosyltransferase 4, Smp3p (132). Different from the mammalian GPI biosynthetic pathway, addition of Man4 is essential for the later steps of GPI biosynthesis (**Table 2**), and is particularly required for transfer of the terminal EtNP by GPI-EtNP transferase 2, a complex of Gpi13p and Gpi11p. Another Man (Man5) is added to Man4 via an α-1,2 or α1,3 linkage by an unidentified enzyme (6, 133). The reaction is carried out in the Golgi apparatus after GPI attachment to proteins, likely via GDP-Man. The functional significance of Man5 is still unclear. The lipid moiety of mature yeast GPI-APs consists of either diacylglycerol containing a very long chain fatty acid [hexacosanoic (C26:0) acid] at the *sn*2 position or ceramide containing phytosphingosine with a very long chain (C26:0) fatty acid (134). The fatty acyl chains in both diacylglycerol and ceramide GPI-APs are sometimes hydroxylated (135, 136). Ceramide structures in GPI anchors are also observed in other species, such as *Aspergillus fumigatus*, *Trypanosoma cruzi*, *Dictyostelium discoideum*, and pear plants (1). Similar to mammalian GPI, the glycan and lipid moieties are remodeled after GPI attachment to proteins in yeast.

LIPID REMODELING OF GPI-APs IN YEAST

The major PI species in yeast are 1-palmitoyl (C16:0)-2-oleoyl (C18:1)-PI and 1-palmitoyl (C16:0)-2-palmitoleoyl (C16:1)-PI (137). GPI is synthesized from such conventional PI species. In an early step of GPI biosynthesis, GlcN-PI is converted to GlcN-(acyl)PI. The acylation is

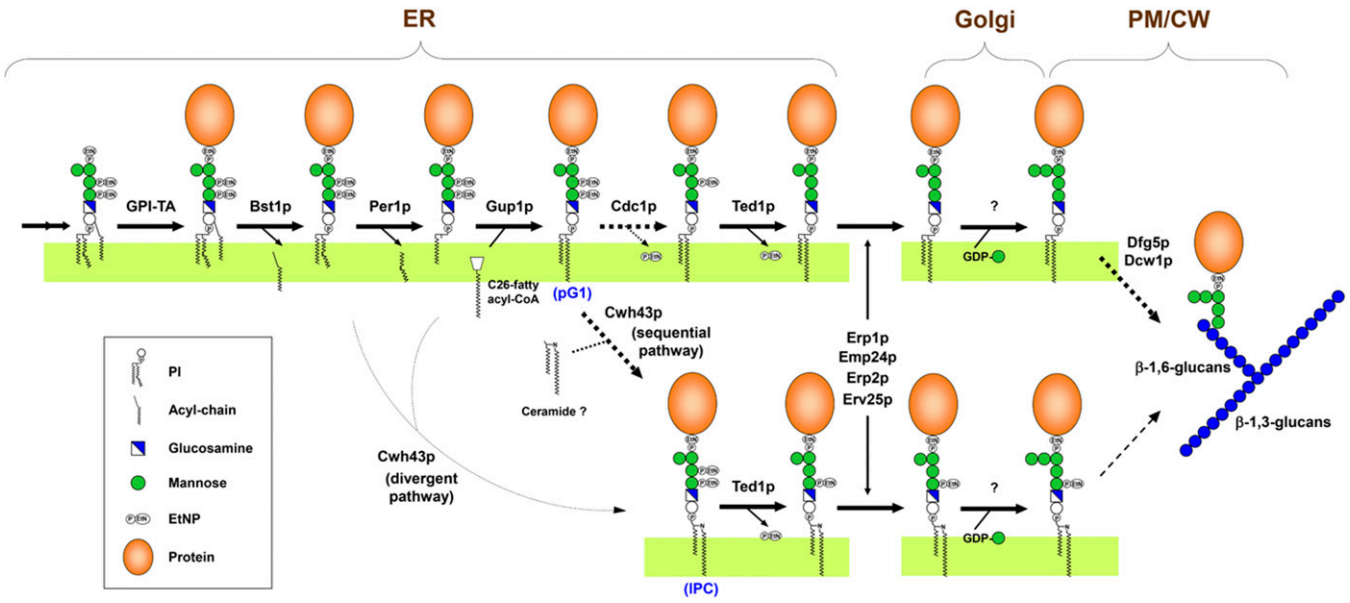

Fig. 5. Remodeling of GPI anchors in yeast *S. cerevisiae*. GPI is synthesized in the ER and transferred to proteins by the GPI-transamidase (GPI-TA) complex. After GPI-attachment to proteins, an acyl-chain linked to inositol is eliminated by Bst1p. Then, an unsaturated fatty acyl chain in the *sn*2 position is removed by Per1p, and a very long saturated (C26:0) fatty acid is reacylated to the position by Gup1p. C26-fatty acyl-CoA is used as the substrate. Many fractions of lipid moieties in GPI anchors are further exchanged from diacylglycerol types to ceramide types. The reaction is mediated by Cwh43p. The substrate for the ceramide remodeling is not clear. A side-chain EtNP attached to Man1 is removed from some fractions of GPI anchors by Cdc1p. It remains unclear which GPI-APs are recognized as the substrates for Cdc1p; whereas, the experimental data suggest that GPI anchors having diacylglycerol types might be the preferential substrates (155). A side-chain EtNP attached to Man2 is removed by Ted1p. The reaction is important for association of GPI-APs with a transport adaptor protein p24 complex. The order of the reactions mediated by Cdc1p and Ted1p is not known. After GPI-APs are transported to the Golgi, additional Man is transferred to the Man4 with α1,2 or α1,3 linkage by unidentified enzymes. On the cell surface, many of GPI-APs are cleaved and cross-linked to β1,6-glucans on the cell wall. Dfg5p and Dcw1p are involved in the cell wall anchorage of GPI-APs. PM/CW, plasma membrane/cell wall. pG1, PI containing a C26-fatty acid at the *sn*2 position.

mediated by Gwt1p, a yeast homolog of PIG-W, as described above (86). After GPI is attached to proteins, the inositol acyl-chain is eliminated by bypass of Sec13 (Bst)1p, a yeast homolog of PGAP1 (71). Subsequently, GPI fatty acid remodeling is carried out in the ER (134, 138, 139). This is different from mammalian cells, where fatty acid remodeling mainly occurs in the Golgi (140) (Figs. 4, 5; Table 2). First, a yeast PGAP3 homolog, Per1p, eliminates an unsaturated fatty acid in the *sn*2 position of GPI anchors (141). Then, C26:0, a very long chain fatty acid, is reacylated to lysoGPI by Gup1p, an MBOAT family member (142). Instead, PGAP2 is involved in GPI acylation in mammalian cells, as described above (80). The N-terminal region of yeast Cwh43p shares homology with PGAP2 (143, 144). However, Cwh43p has an additional C-terminal domain consisting of about 700 amino acids, which shares characteristics with endonuclease, exonuclease, and phosphatase proteins. The C domain is also observed in inositol phosphingolipid-specific phospholipase C, Isc1p, and the phosphatidylinositide 5-phosphatases, Inp51/52/53/54p. In *cwh43*Δ cells, GPI with diacylglycerol consisting of a very long chain fatty acid at the *sn*2 position was accumulated and ceramide type GPI anchors were completely lost, suggesting that Cwh43p is required for ceramide remodeling of GPI anchors (Fig. 5; Table 2) (143, 144). The substrate for ceramide remodeling is not clear. Interestingly, yeast lacking all known ceramide synthases still make almost normal ceramide-type GPI anchors, whereas yeast lacking Ser palmitoyltransferase do not produce ceramide-type GPI anchors (145). Therefore, it is possible that there is an unidentified pathway used to produce ceramide that is preferentially incorporated into GPI anchors. Two models have been proposed to describe the lipid remodeling pathway of GPI anchors in yeast, the sequential pathway and the divergent pathway (143, 144). In the former model, PI moieties in GPI anchors are sequentially modified from conventional PI to lysoPI, then a C26:0 fatty acid chain is added at the *sn*2 position (pG1) to finally generate inositol-phospho-ceramides (IPCs). The divergent pathway model involves lysoPI generated by the reaction of Per1p as a substrate for two separated reactions by Gup1p and Cwh43p, generating pG1-type and IPC-type GPI

TABLE 2. Differences in GPI biosynthesis/remodeling in mammals and yeast

Reaction	Mammals	Yeast
PI remodeling	Diacyl PI to alkyl-acyl/diacyl PI	Diacyl PI to ceramide by Cwh43p
Man4 addition by PIG-Z/SMP3	Not essential	Essential
Fatty acid remodeling	Reaction in Golgi by PGAP3 and PGAP2	Reaction in ER by Per1p and Gup1p
Man1-linked EtNP-side chain	Present in GPI-AP	Removed in ER by Cdc1p

anchors, respectively. According to a recent study, the sequential pathway is the main pathway for lipid remodeling, but direct conversion pathways from conventional PI and lysoPI in GPI anchors to IPCs likely exist (146).

REMODELING OF SIDE-CHAIN EtNPs ON GPI-GLYCAN IN YEAST

During GPI biosynthesis in yeast, three EtNPs are added to the GPI structure, similar to what occurs in mammalian cells. PE seems to be used as the donor substrate (147, 148). Mcd4p, a complex of Gpi13p and Gpi11p, and a complex of Gpi7p and Gpi11p transfer EtNP to the 2-position of Man1, the 6-position of Man3, and the 6-position of Man2, respectively. The EtNP on Man3 added by Gpi13p/Gpi11p is used for linking to proteins. In yeast, two other side-chain EtNPs seem to be important for the regulation of GPI lipid remodeling, GPI-AP transport and cell wall localization. *MCD4* is an essential gene because the presence of EtNP on Man1 in the GPI intermediate is critical for the subsequent mannosylation (addition of Man3) by Gpi10p (149). However, the essentiality of *MCD4* is cancelled by overexpression of *Trypanosoma brucei GPI10*, which does not require the side-chain EtNP for substrate recognition (150). Incorporation of ceramide into GPI was impaired in *mcd4*Δ/*T. brucei GPI10* mutant cells. In *gpi7* mutant cells, ceramide remodeling was also partially impaired (151). *GPI7* is a nonessential gene, but the mutant showed cell wall defects and growth and cell separation deficiencies at high temperature (151, 152). This might be due to a GPI anchoring inefficiency, defects in ceramide remodeling and/or slow transport of GPI-APs. These results suggest that ceramide remodelase, Cwh43p, recognizes side-chain EtNPs on Man1 and Man2 in GPI anchors.

Recently, genes involved in the removal of side-chain EtNPs from GPI-APs were identified in yeast. Both *TED1* and *CDC1* are homologs of mammalian PGAP5, which is required for the removal of a side-chain EtNP from the Man2 in the GPI anchors (Table 2) (72). The *ted1*Δ mutant cells showed delayed ER-to-Golgi transport of a GPI-AP, Gas1p (153), but the effect was rescued by mutation in *GPI7*, suggesting that Ted1p functions as an enzyme required for removal of an EtNP from Man2 similar to PGAP5 (Fig. 5) (154). The elimination of the side-chain EtNP from Man2 by Ted1p is critical for recognition by the p24 complex (Emp24p, Erv25p, Erp1p, and Erp2p), transport adaptors for GPI-APs, as described below. The other PGAP5 homolog, Cdc1p, is thought to remove a side-chain EtNP from Man1 (Fig. 5) (155). This elimination occurs in a fraction of GPI-APs. In contrast to *ted1*Δ mutant cells, transport of GPI-APs was almost normal in *cdc1* mutant cells. Instead, the reaction mediated by Cdc1p might be related to cell wall anchorage of GPI-APs, as described below.

YEAST GPI-AP TRANSPORT FROM THE ER AND BST GENES

After GPI-APs are synthesized in the ER, they are transported to the Golgi. In yeast, GPI-APs are sorted into the specific ERESs, segregated and transported to the Golgi with COPII vesicles distinct from those used for transport of other secretory proteins, such as Gap1p, Hxt1p, and ALP (156–158). Sorting of GPI-APs does not require COPII proteins, but structural remodeling of GPI is critical for ER-to-Golgi transport (154, 158). Deletion of GPI remodeling genes, including *BST1*, *PER1*, *GUP1*, or *TED1*, causes delay in transport of GPI-APs from the ER to the Golgi (141, 142, 153, 159, 160). It is thought that the remodeled GPI-APs and ceramides, both of which possess a very long chain fatty acid, form specific domains at the ER and are cooperatively sorted to the specific ERESs (157, 158). The GPI-APs preassembled at the ERESs are transported from the ER by COPII vesicles. Because GPI-APs are luminally localized and cannot interact with the COPII component directly, adaptors that link GPI-APs and COPII are required to ensure efficient transport. Similar to mammalian cells, the yeast p24 protein complex acts as an adaptor for incorporation of GPI-APs into the COPII vesicles (161). It has been shown that the p24 proteins preferentially interact with lipid-remodeled GPI-APs (157, 162, 163). Recently, it was shown that the p24 proteins possess a lectin activity which allows them to bind GPI-glycan lacking side-chain EtNPs, the structure modified by Ted1p (154). The cytosolic tails of p24 proteins are used for binding with COPII components. Of the COPII components, members of the Sec24 protein family directly recognize the ER export signal in cargoes and cargo adaptors (164). In yeast, there are three members of the Sec24 family: Sec24p, Lst1p, and Iss1p. Lst1p recognizes the cytosolic region of p24 proteins (157, 165). Consistent with this, Lst1p is required for efficient transport of GPI-APs (166). In vitro analysis suggests that Lst1p is used to produce COPII vesicles for packaging relatively large and/or clustered cargoes (167). Because GPI-APs are clustered depending upon the types of lipids at the ERESs, usage of Lst1p is in line with the COPII formation for GPI-APs.

Although the function of the yeast p24 complex is similar to that of mammals, there is a difference in their GPI-AP sorting. The yeast p24 complex is not involved in cargo concentration, but is recruited to preconcentrated GPI-APs that are already sorted into specific ERESs in a lipid-dependent manner (154, 156). In contrast, mammalian p24 proteins are required for sorting of GPI-APs into the ERESs (73). Therefore, the yeast p24 complex functions as an "adaptor" that links GPI-APs and COPII; whereas, mammalian p24 proteins act as "cargo receptors" for GPI-APs to sort into the ERESs. The difference is caused by the lipid structures of GPI-APs in the ER between yeast and mammals. In mammalian cells, because protein-bound GPI at the ER contains PI bearing an *sn*2-linked unsaturated fatty acid and the GPI fatty acid remodeling occurs later in the Golgi, GPI-APs are not sorted by spontaneous interaction of lipids (140).

Protein parts anchored by GPI are localized in the lumen of the ER and are highly packed into the specific ERESs, resulting in steric pressure on the lumen of the ER. This asymmetrical distribution confers opposing curvature on the membrane. Therefore, COPII vesicle formation

from GPI-AP-concentrated ERESs requires a strong force for the membrane to bend against the opposing curvature. Sec13p, one of the COPII components, forms a complex with Sec31p to generate the outer layer of the COPII coat and increases the rigidity to drive membrane bending (168, 169). A gene encoding GPI inositol deacylase, *BST1*, was originally isolated as the responsible gene for the mutant that cancels the lethality of *sec13* mutant yeast, called the *BST* gene (170). From the same genetic screening, *EMP24* (*BST2*) and *ERV25* encoding p24 proteins were isolated as *BST* genes. Through contentious and comprehensive analyses, it was revealed that *PER1*, *GUP1*, *TED1*, *ERP1*, *ERP2*, *YGL024w*, and *ERV29* all function as *BST* genes in addition to *BST1*, *EMP24*, and *ERV25* (169, 171). All genes except *YGL024w* and *ERV29* encode proteins that are required for GPI-AP remodeling and transport (Fig. 5). Without Sec13p, COPII could not obtain sufficient rigidity to drive membrane bending against GPI-AP-enriched domains (163). From the analysis of mutations of genes involved in GPI-AP remodeling, it was concluded that GPI-APs are not concentrated or sorted at the ERESs. Thus, COPII vesicles are formed without Sec13p, which would bypass the lethality caused by the *SEC13* gene mutation (169). These results also support the ideas that structural remodeling of GPI-APs and their lipid-dependent sorting are critical for the efficient transport from the ER.

RELATIONSHIP BETWEEN GPI REMODELING AND CELL WALL ANCHORAGE

The yeast cell wall consists of chitin, glucan, and mannan layers. GPI-APs are the major components of the mannan layer (172). After GPI-APs reach the plasma membrane, many yeast GPI-APs are further transported to the cell wall (Fig. 5). It is thought that the GPI moiety is cleaved between Man1 and GlcN, and is cross-linked to the β1,6-glucans in the cell wall (172, 173). Dfg5p and Dcw1p are putative mannosidases involved in the cell wall anchorage of GPI-APs (139, 174, 175). It is still unclear whether the cleavage of GPI and its transfer to the cell wall occur in a one-step trans-glycosylation or in multiple separate reactions.

To date, it is not clear which GPI-AP molecules are transferred to the cell wall. The amino acid sequences near the GPI anchors influence the localization of GPI-APs. If two basic amino acids are located in the ω(−) region (where the ω-site is the amino acid that is modified by GPI), then most proteins are retained in the plasma membrane (176–178). However, this consensus sequence might not be generalized. Many yeast GPI-APs contain a Ser/threonine-rich region near the GPI anchors, which becomes *O*-mannosylated in most cases. The presence of the Ser/threonine-rich region abolishes the effect of the basic amino acids to retain GPI-APs at the plasma membrane (179). Furthermore, some GPI-APs seem to be localized both at the plasma membrane and the cell wall.

Analysis of *cdc1* mutant cells led to the hypothesis that GPI-APs bearing a diacylglycerol-type GPI containing a very long chain fatty acid and lacking the side-chain EtNPs are efficiently anchored to the cell wall (Fig. 5) (155). The *cdc1* mutant cells showed defects in their cell wall. Additionally, GPI-APs localized in the cell wall fraction were not always cross-linked with β1,6-glucans and were easily extracted, but were probably trapped in the meshwork of the cell wall in *cdc1* mutant cells (155). When GPI-APs are cross-linked with β1,6-glucans on the cell wall, the hydroxyl group at the 1-position of Man1 would form the glycosidic bond. It is possible that persistence of the side-chain EtNP, which is linked to the 2-position on Man1, interferes with the efficient transfer of GPI-APs onto the β1,6-glucans. Alternatively, the removal of EtNP from Man1 by Cdc1p might be required for the correct cleavage of GPI-APs at the plasma membrane. In *cdc1* mutant cells, the amount of ceramide-type GPI was normal, but a 3- to 4-fold accumulation of a type of diacylglycerol GPI containing a very long chain fatty acid was observed compared with wild-type cells (155). The reason why GPI-APs were trapped on the cell wall meshwork in *cdc1* mutants might be because GPI cleavage occurred at an abnormal position to compensate for the cell wall defects in *cdc1* mutants. Although these hypotheses and ideas, where Cdc1p acts on diacylglycerol-type GPI anchors and the elimination of the EtNP somehow becomes the marker for GPI-APs to transfer to the cell wall, are very interesting, further work is required before conclusions can be made.

INHIBITORS OF GPI BIOSYNTHETIC PATHWAYS

GPI-APs are major components of the mannan layer in the yeast cell wall and are critical for cell wall integrity and cell adhesion in fungi. GPI biosynthesis is essential for yeast growth and virulence of infectious fungi (134, 180, 181). Therefore, the biosynthesis of GPI has the potential to be targeted for the development of anti-fungal agents. To date, several inhibitors targeting GPI have been reported. Treatment of mannosamine appears to block the addition of Man3 to the GPI precursor in both mammalian cells and African trypanosomes (182–184). The Ser protease inhibitor, PMSF, and diisopropyl fluorophosphate block the GPI inositol acylation in African trypanosomes; whereas, PMSF does not affect mammalian GPI biosynthesis (185, 186). It is not known whether PMSF can inhibit yeast GPI biosynthesis. However, these inhibitors are not specific for GPI biosynthesis. In contrast, a terpenoid lactone, YW3548, which is a metabolite from *Codinea simplex*, was reported to be an inhibitor of the GPI biosynthetic pathway (187). The compound seems to specifically inhibit GPI biosynthesis. YW3548 inhibits the step where EtNP is added to Man1 in the GPI precursor by yeast Mcd4p and mammalian PIG-N (78). Although YW3548 is a very good tool to analyze GPI biosynthesis, it cannot be applied as an anti-fungal reagent. Another inhibitor for GPI biosynthesis, 1-[4-butylbenzyl]isoquinoline (BIQ), has also been reported. BIQ blocks the GPI inositol acylation mediated by yeast Gwt1p (86, 188). Furthermore, using BIQ as a starting point, a new compound, E1210, was

found to have improved antifungal activity (189). E1210 is a broad spectrum antifungal agent against different pathogenic fungi, including *Candida*, *Aspergillus*, *Fusarium*, and *Scedosporium* species (190–193). Importantly, the compound seems to be fungal specific and does not inhibit mammalian GPI inositol acylase PIG-W activity (194). In vivo efficacy of this agent has been demonstrated after oral administration in a murine model against candidiasis, aspergillosis, and fusariosis (189, 195). Another group reported that a different compound, gepinacin, also inhibited Gwt1p activity (196). There are several differences in GPI biosynthesis and remodeling between mammalian cells, fungi, and protozoan parasites. Therefore, development of inhibitors focusing on this pathway may prove useful as anti-fungal and anti-protozoan agents.

FUTURE PERSPECTIVE

There are a number of outstanding issues to be clarified in the biosynthetic pathway of mammalian GPI-APs (1). Genes involved in generation of the Sia-Galβ3-GalNAcβ4 side branch linked to Man1 are not identified and biological functions of this side branch and the Man4 side branch found in some GPI-APs are totally unknown (2). Mechanisms of flip of GlcN-PI from cytoplasmic face to luminal face in the ER needs to be clarified (3). Biological functions and enzymatic mechanisms of GlcN-(acyl)PI lipid remodeling, in which the diacyl form is remodeled to a mixture of 1-alkyl-2-acyl and diacyl forms in the ER, await clarification (4). Quality control of GPI-AP biosynthesis includes coordination of protein folding and remodeling of the GPI moiety before exit from the ER. There might be additional mechanisms in GPI-AP quality control that are not involved in that of other secretory proteins. Elucidation of these and other outstanding issues should contribute to mechanistic understanding of disorders caused by defective GPI-AP biosynthesis.

Studies with yeast have led to clarification of genetic, biochemical, and cell biological mechanisms of GPI-AP biosynthesis. There are several issues yet to be clarified in yeast GPI-AP biosynthesis. The exact mechanisms of GPI lipid remodeling that generate diacyl and ceramide forms of GPI-APs need to be clarified, as does the biological significance of the two forms of lipid moiety. Understanding these issues should contribute to developing effective measures to control fungal diseases and novel protein biotechnology.

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