Changes in molecular species profiles of glycosylphosphatidylinositol anchor precursors in early stages of biosynthesis

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Abstract Glycosylphosphatidylinositol (GPI) anchor is a major lipidation in posttranslational modification. GPI anchor precursors are biosynthesized from endogenous phosphatidylinositol (PI) species and attached to proteins in the endoplasmic reticulum. Endogenous PIs are characterized by dominance of diacyl species and the presence of polyunsaturated fatty acyl chain, such as 18:0-20:4, at the sn-2 position. In contrast, the features of mammalian glycosylphosphatidylinositol-anchored proteins (GPI-APs) are domination of alkyl/acyl PI species and the presence of saturated fatty acyl chains at the sn-2 position, the latter being consistent with association with lipid rafts. Recent studies showed that saturated fatty acyl chain at sn-2 is introduced by fatty acid remodeling that occurs in GPI-APs. To gain insight into the former feature, we analyzed the molecular species of several different GPI precursors derived from various mammalian mutant cell lines. Here, we show that the PI species profile greatly changed in the precursor glucosamine (GlcN)-acyl-PI and became very similar to that of GPI-APs. Therefore, a specific feature of the PI moieties of mature GPI-APs, domination of alkyl (or alkenyl)/acyl species over diacyl acyl chain as the major PI species, is introduced by fatty acid remodeling. They had alkyl (or alkenyl)/acyl types with unsaturated acyl chain as the major PI species. Changes in molecular species profiles of glycosylphosphatidylinositol anchor precursors in early stages of biosynthesis. J. Lipid Res. 2007. 48: 1599–1606.

Supplementary key words lipid molecular species • fatty acyl chain remodeling • mass spectrometry • liquid chromatography-electrospray ionization-mass spectrometry • phospholipid localization

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Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are attached to the plasma membrane in all eukaryotes (1–3). Glycosylphosphatidylinositol (GPI) anchors are normally composed of three to four mannoses, a glucosamine (GlcN), two to three phosphorylethanolamines, and an inositol phospholipid, and the ethanolamine amino group is bound to the C terminus of GPI-AP (4, 5). GPI is one of the many posttranslational modifications that regulate various functions, such as intracellular localization and signal transduction (3–10). GPIs are biosynthesized at the endoplasmic reticulum membrane in eight or nine steps and then transported to the plasma membrane via the Golgi apparatus (11). Many genes related to GPI biosynthesis, such as PIGA and PIGM, have been cloned (12, 13). It is widely known that mutations in the PIGA gene cause paroxysmal nocturnal hemoglobinuria, a clonal hematopoietic stem cell disease (14).

Many GPI-APs aggregate at lipid rafts, which are sphingolipid- and cholesterol-enriched microdomains on the plasma membrane, and play important roles in signal transduction (3–10). In mammalian cells, phosphatidylinositol (PI) moieties in mature GPI-APs contain saturated or monounsaturated fatty acyl chains at the sn-2 position, although PIs in general cells are polyunsaturated molecular species such as 20:4. Previous reports have shown that GPI-APs are matured by remodeling of their PI moieties (1, 15–18). It was also found recently that PGAP2 (for Post-GPI Attachment to Proteins 2), which mainly exists in the Golgi, is involved in fatty acid remodeling. However, there are many remodeling mechanisms that have

Abbreviations: alk, alkyl/acyl or alkenyl/acyl; f.u., fatty acyl residue; GlcN, glucosamine; GlcNAc, N-acetyl glucosamine; GPI, glycosylphosphatidylinositol; GPI-AP, glycosylphosphatidylinositol-anchored protein; Ins-P, inositol phosphate; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; PGAP, Post-GPI Attachment to Proteins; PI, phosphatidylinositol.

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not yet been elucidated (19). In a previous report, we used liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) for analysis of GPI precursors trapped in several mutant cells during the early steps of GPI biosynthesis. ESI-MS with the feature of soft ionization is usually used for profiling of molecular weight-related ions (20, 21). We also attempted to elucidate the precise structures of the GPI precursors by MS/MS analysis. Here, we conducted a precise comparison of the compositions of GPI precursor species during the early biosynthetic steps.

MATERIALS AND METHODS

Cells and materials

All solvents used were MS grade and were purchased from Wako Pure Chemicals (Osaka, Japan). Deionized water was obtained using a Milli-Q water system (Millipore, Milford, MA). All cell lines used were derivatives of CHO cells. Wild-type 3B2A cells (22) were cultured in Ham’s F12 medium (Sigma). All mutant cell lines [M2S2 (accumulating N-acetyl glucosamine-PI; GlcNAc-PI) (22), CHOPA10.14 (accumulating GlcN-PI) (23), and Lec15 (accumulating GlcN-acyl-PI) (24)] were cultured in Ham’s F12 medium supplemented with 10% fetal calf serum and 600 μg/ml G418.

Extraction of phospholipids

Wild and mutant CHO cells (1 × 10⁸) were homogenized with methanol, and the lipid mixture was extracted using the method of Bligh and Dyer (25). The total lipid extract was dried under a gentle stream of nitrogen and then dissolved in 100 μl of chloroform-methanol (1:1).

ESI-MS analysis of phospholipids

ESI-MS analysis was performed using a quadrupole time-of-flight hybrid mass spectrometer, Q-TOF II or Q-TOF micro™ (Micromass, Manchester, England), and an Ultimate HPLC system combined with a FAMOS autosampler (LC-Packings, San Francisco, CA). The mass range of the instrument was set at m/z 400-1,400. Spectra were recorded in positive and negative ion modes. Capillary voltage was set at 3.0 kV, cone voltages at 30 V, and source block temperatures at 100°C. The collision gas used for MS/MS experiments was argon (pAr = 7.5 × 10⁻⁵ mbar), and the collision energy was set at 50 V.

RESULTS

Comparison of molecular species at each biosynthetic step of GPI

GPI anchors are synthesized from intracellular PI [(i) in Fig. 1] by sequential additions of hexoses, an acyl chain, and phosphorylethanolamines. First, GlcNAc [(ii)] from UDP-GlcNAc is added to PI. Next, GlcNAc-PI is deacetylated to form glucosamine-PI (GlcN-PI) [(iii)]. Then, GlcN-PI is transferred to the lumen side by flip-flop transfer and converted to GlcN-acyl-PI [(iv)] by palmitoylation at C-2 of inositol. Subsequently, three mannoses and two phosphorylethanolamines are added to GlcN-acyl-PI, generating the complete GPI anchor (Fig. 1).

Endogenous PIs are dominant in 18:0-20:4 diacyl PI. In contrast, the features of mammalian GPI-anchored proteins are domination of alkyl (or alkenyl)/acyl (alk/acyl) PI species and the presence of saturated fatty acyl chains at the sn-2 position. To determine structures of PI moieties in early GPI precursors, the lipid mixture extracted from each biosynthetic mutant cell was subjected to LC-ESI-MS analysis. GPI precursors, which are minor components,
were detected with LC-ESI-MS by separating from major phospholipids. For example, GlcNAc-PIs were eluted before PIs and other phospholipids (Fig. 2). In the original precursor (PI), the major molecular species was 38:4 (18:0-20:4) diacyl type, with other species present only in small amounts (Fig. 3A). In the second (GlcNAc-PI) and third (GlcN-PI) precursors, the 38:4 diacyl type was also a major species; the composition of molecular species was almost unchanged up to the third precursor, although some minor species were increased (Fig. 3B, C). However, in the fourth precursor (GlcN-acyl-PI), not only 38:4 diacyl species but also many other molecular species, such as 38:4 alk/acyl, were detected as major components (Fig. 3D). In this process, molecular species containing longer acyl chain also seem to be accumulated. This result showed that the alkyl or alkenyl binding type of GPI molecule increased greatly between the third and fourth precursors. The high content of alk/acyl PI in GlcN-acyl-PI was also found in a DPM1-defective mutant of mouse T-cell lymphoma cells, BW5147Thy-1−/− (data not shown), suggesting that dominance of alk/acyl types in GlcN-acyl-PI is common in mammalian cells. It was a common characteristic up to the fourth step that most GPls had unsaturated fatty acyl chains (Fig. 4).

Identification of each GPI precursor species

To assign the peaks shown in Fig. 3, we performed LC-ESI-MS/MS and identified the fatty acid-related residues and GPI precursor-specific fragments. For the first GPI precursor, the peaks at m/z 885 was identified as 18:0-20:4 diacyl PI, which is the most common molecular species in mammalian cells (data not shown).

In Fig. 5, the fragment peak at m/z 444 was derived from GlcNAc inositol phosphate ([GlcNAc-Ins-P]−, C14H23O13NP; Fig. 5A); thus, it was confirmed that the peak at m/z 1,088 and 1,052 in Fig. 3B were GlcNAc-PI. The peak at m/z 1,088 was identified as 18:0-20:4 diacyl by the fatty acyl residues 18:0 (C18H35O2, m/z 283) and 20:4 (C20H31O2, m/z 303) [Fig. 5B, (i)], and the peak at m/z 1,052 was identified as 18:0-18:1 alk/acyl by the fatty acyl residue 18:1 (C18H33O2, m/z 281) [Fig. 5B, (ii)].

In Fig. 6, the fragment peak at m/z 402 was derived from GlcN-cyclic-inositol phosphate ([GlcN-Ins-P]−, C12H21O12NP; Fig. 6A); thus, it was confirmed that the peaks at m/z 1,072 and 1,046 in Fig. 3C were GlcN-PI. The peak at m/z 1,072 was identified as an 18:0-22:5 diacyl species by...
the fatty acyl residues 18:0 (C\textsubscript{18}H\textsubscript{35}O\textsubscript{2}, m/z 283) and 22:5 (C\textsubscript{22}H\textsubscript{33}O\textsubscript{2}, m/z 329) and the loss of fatty acyl residue [M – f.a.22:5]\textsuperscript{−} (C\textsubscript{33}H\textsubscript{61}O\textsubscript{15}NP, m/z 742) [Fig. 6B, (i)], and the peak at m/z 1,046 was identified as an 18:0-20:4 diacyl species by the fatty acyl residues 18:0 (C\textsubscript{18}H\textsubscript{35}O\textsubscript{2}, m/z 283) and 20:4 (C\textsubscript{20}H\textsubscript{31}O\textsubscript{2}, m/z 303) and [M – f.a.20:4]\textsuperscript{−} (C\textsubscript{33}H\textsubscript{61}O\textsubscript{15}NP, m/z 742) [Fig. 6B, (ii)].

In Fig. 7, the fragment peak at m/z 640 was derived from GlcN-acyl-cyclic-inositol phosphate ([GlcN-Ins(-palmitoyl)-P]\textsuperscript{−}, C\textsubscript{28}H\textsubscript{51}O\textsubscript{13}NP; Fig. 7A); thus, it was confirmed that the
peaks at m/z 1,284, 1,268, 1,270, and 1,242 in Fig. 3D were GlcN-acyl-PI. In addition, it was confirmed that these species contained a palmitoyl chain at the C-2 of inositol, because a 16:0 fatty acyl residue (C_{16}H_{31}O_{2}, m/z 255) was detected. The peak at m/z 1,284 was identified as 18:0-20:4 diacyl by the fatty acyl residues 18:0 and 20:4 (C_{18}H_{35}O_{2}, m/z 283 and C_{20}H_{31}O_{2}, m/z 303), lyso-phosphatidic acid 18:0 (C_{21}H_{43}O_{7}P, m/z 423), cyclic phosphatidic acid 18:0 (C_{21}H_{41}O_{6}P, m/z 405), and [M-2f.a.20:4]^{2} (C_{49}H_{91}O_{16}NP, m/z 980) [Fig. 7B, (i), 7C, (i)]. Furthermore, the peak at m/z 1,284 contained not only 18:0-20:4 diacyl species but also 16:0-22:4 diacyl species, because the fatty acyl residues 16:0 and 22:4 (C_{16}H_{31}O_{2}, m/z 255 and C_{22}H_{31}O_{2}, m/z 331) and [M-2f.a.22:4]^{2} (C_{47}H_{87}O_{16}NP, m/z 952) were detected as fragment ions. Similarly, the peaks at m/z 1,268, 1,270, and 1,242 in Fig. 3D were identified as 18:1-20:4 alk/acyl, 18:1-20:3 alk/acyl, and 16:0-20:4 alk/acyl species (major species). In addition to these GlcN-acyl-PI species with palmitoyl chains, GlcN-Acyl-PI species with myristoyl chains were also identified based on the presence of [GlcN-Ins(-myristoyl)-P]^{2} (C_{26}H_{47}O_{13}NP, m/z 612) and a 14:0 fatty acyl residue as fragment ions (C_{14}H_{27}O_{2}, m/z 227) in these experiments, many molecular species with the same mass value but with different pairs of fatty chains were found to exist in the GlcN-acyl-PI accumulated mutants (Table 1).

**DISCUSSION**

Previous reports have shown that saturated fatty acyl chains in mature GPI-APs are important for integration into the lipid rafts on the plasma membrane and that there are few GPI-APs with high unsaturated fatty chains (1, 15–18). It was also reported that large amounts of alkyl/acyl-type PI are contained in mature GPI-APs (16). However, the molecular species of intracellular PI, which is the original precursor of the GPI anchor, is generally an 18:0-20:4 diacyl species in mammalian cells. These results indicate that GPI species are matured through fatty chain remodeling and/or enzymatic selection; however, clear experimental evidence for both mechanisms had not been presented. Recently, Maeda et al. (26) found that GPI-APs were remodeled from PUFAs (e.g., 20:4) to 18:0 by PGAP2 and PGAP3 in the Golgi apparatus and that only mature GPI-APs were transported to rafts, reinforcing the idea that the fatty acid moiety of GPI-APs is particularly important for their precise localization and function in the membrane (Fig. 1).

Here, we investigated the alteration in profile of several GPI precursor species using LC-ESI-MS/MS. This technique has been shown to be very effective in lipid analysis, and MS/MS mode is used broadly for identification (27–29). The lipid mixtures extracted from CHO mutant cells at the early stages of GPI biosynthesis were investigated using this method. In their MS/MS spectra, the GPI precursor-specific fragments (e.g., GlcNAc-PI: [GlcNAc-Ins-P]^{−} ; m/z 444) and fatty acid chain residues (e.g., 18:0-20:4: [f.a. 18:0]^{−}; m/z 283 and [f.a. 20:4]^{−}; m/z 303) were detected and identified. In the case of diacyl species, two fatty acid chain residues were detected [Fig. 5B, (i)], whereas for alkyl or alkenyl binding types, only one fatty acyl chain residue was detected [Fig. 5B, (ii)], because the fatty chain residue was not fragmented from alkyl or alkenyl binding at the sn-1 position.

Comparison of the GPI precursor species showed that their profiles were almost identical up to the third precursor of biosynthesis (GlcN-PI), although the amounts of alk/acyl species were slightly higher in GlcN-PI and GlcN-PI than in the initial PI. However, we observed a marked increase between the third and fourth (GlcN-acyl-PI) precursors (Fig. 7). A similar increase in alk/acyl types was seen in murine lymphocytes, suggesting that it is a general phenomenon in mammalian cells. There is a report that ~15% of GlcN-acyl-PI from HeLa S3 cells was
Fig. 7. Structural elucidation of GlcN-acyl-PI species from specific fragments by LC-ESI-MS/MS. The lipid mixture from CHO mutant cells (Lec15) accumulating GlcN-acyl-PI was analyzed by LC-ESI-MS/MS. The fragment peak at \( m/z \) 640 is the \([\text{GlcN-Ins-palmitoyl (Pal)-P}]^-\) ion, and that at \( m/z \) 612 is the \([\text{GlcN-Ins-myristoyl (Myr)-P}]^-\) ion, which is derived from the core structure of GlcN-acyl-PI. A: Fragmentation pattern. B: MS/MS of (i), \( m/z \) 1,284 (38:4 diacyl); (ii), \( m/z \) 1,268 (38:5 alk/acyl); (iii), \( m/z \) 1,270 (38:4 alk/acyl); and (iv), \( m/z \) 1,242 (36:4 alk/acyl). C: Spectrum shown in B expanded between \( m/z \) 200 and 500. cPA, cyclic phosphatidic acid; LPA, lyso-phosphatidic acid.
TABLE 1. List of glucosamine-acyl-phosphatidylinositol species identified by liquid chromatography-electrospray ionization-tandem mass spectrometry

<table>
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<tr>
<th>m/z</th>
<th>Palmityl</th>
<th>Myristoyl</th>
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<tr>
<td>1,284</td>
<td>38:4</td>
<td>40:4</td>
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<td>1,270</td>
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<td>1,242</td>
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GlcN-acyl-PI contained only palmitic acid at this position in mammalian cells (30). However, based on our detection of [GlcN-Ins(-myristoyl)-P]− fragments (m/z 612) (Fig. 7B), we conclude that myristic GlcN-acyl-PIs may also exist. 

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REFERENCES


