

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

Toshiaki Houjou,<sup>\*</sup> Jun Hayakawa,<sup>1,†</sup> Reika Watanabe,<sup>2,§</sup> Yuko Tashima,<sup>§</sup> Yusuke Maeda,<sup>§</sup> Taroh Kinoshita,<sup>§</sup> and Ryo Taguchi<sup>3,\*,†</sup>

Graduate School of Medicine,<sup>\*</sup> University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan; Nagoya City University,<sup>†</sup> Nagoya-shi, Aichi 467-8603, Japan; and Research Institute for Microbial Diseases,<sup>§</sup> Osaka University, Suita-shi, Osaka 565-0871, Japan

**Abstract** Glycosylphosphatidylinositol (GPI) anchor is a major lipidation in posttranslational modification. GPI anchor precursors are biosynthesized from endogenous phosphatidylinositols (PIs) and attached to proteins in the endoplasmic reticulum. Endogenous PIs are characterized by domination 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 before fatty acid remodeling. They had alkyl (or alkenyl)/acyl types with unsaturated acyl chain as the major PI species. Therefore, a specific feature of the PI moieties of mature GPI-APs, domination of alkyl (or alkenyl)/acyl type species over diacyl types, is established at the stage of GlcN-acyl-PI.—Houjou, T., J. Hayakawa, R. Watanabe, Y. Tashima, Y. Maeda, T. Kinoshita, and R. Taguchi. **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

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/acyl, alkyl/acyl or alkenyl/acyl; f.a., 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.

<sup>1</sup> Present address of J. Hayakawa: Shionogi Co., Ltd., 3-1-8 Docyu-machi, Cyuoh-Ku, Osaka Japan.

<sup>2</sup> Present address of R. Watanabe: Department of Biochemistry, Sciences II, 30 Quai E. Ansermet, CH-1211 Geneva 4, Switzerland.

<sup>3</sup> To whom correspondence should be addressed.

e-mail: rytagu@m.u-tokyo.ac.jp

Manuscript received 22 February 2007 and in revised form 30 March 2007.

Published, JLR Papers in Press, April 20, 2007.

DOI 10.1194/jlr.M700095-JLR200

Copyright © 2007 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at <http://www.jlr.org>

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  $\mu\text{g}/\text{ml}$  G418.

### Extraction of phospholipids

Wild and mutant CHO cells ( $1 \times 10^8$ ) 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  $\mu\text{l}$  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 ( $p_{\text{Ar}} = 7.5 \times 10^{-5}$  mbar), and the collision energy was set at 50 V.

### LC-ESI-MS

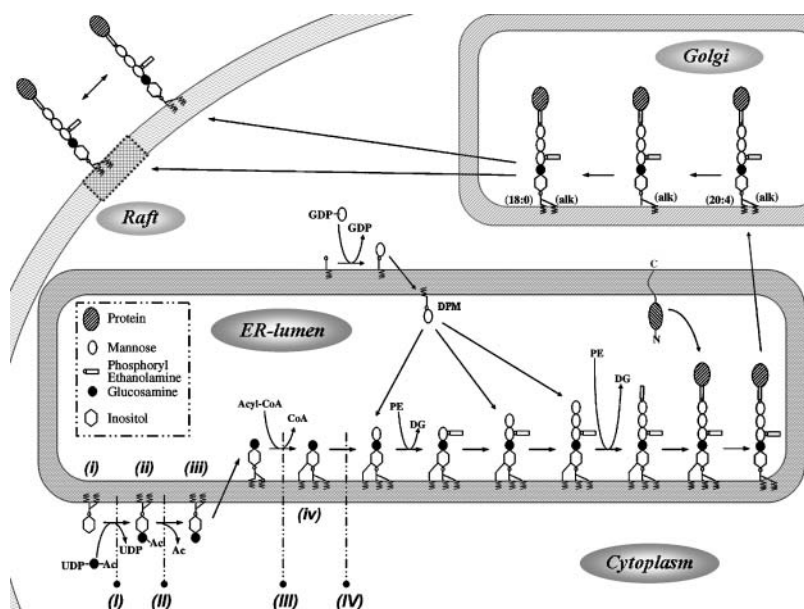
LC separation was carried out using a Si60 (Nomura Chemicals) normal phase LC column ( $150 \times 0.3$  mm inner diameter) at room temperature. The mobile phase composition was acetonitrile-methanol-water (18:11:1; 0.1% ammonium formate, pH 7.8). The mobile phase was pumped at a flow rate of 3  $\mu\text{l}/\text{min}$  for isocratic elution. Typically, 3  $\mu\text{l}$  of sample was used.

## RESULTS

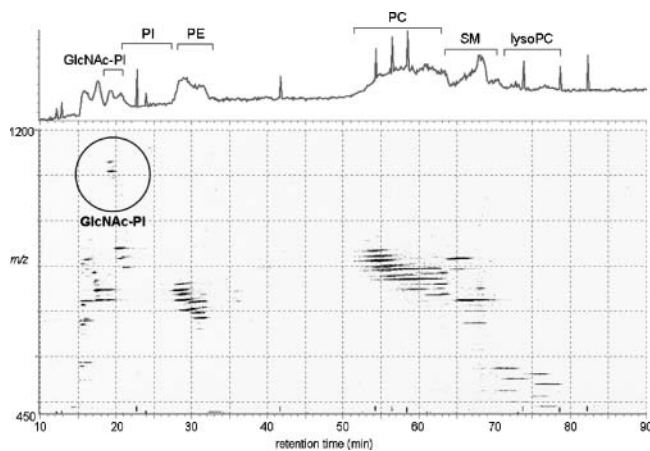
### Comparison of molecular species at each biosynthetic step of GPI

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 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,



**Fig. 1.** Biosynthetic pathway of glycosylphosphatidylinositol-anchored proteins (GPI-APs). Glycosylphosphatidylinositol (GPI) precursors are biosynthesized at the endoplasmic reticulum membrane. First, *N*-acetyl glucosamine [GlcNAc; (ii)] from UDP-GlcNAc is added to phosphatidylinositol [PI; (i)]. Next, GlcNAc-PI is deacetylated to form glucosamine-PI [GlcN-PI; (iii)]. Then, GlcN-PI undergoes a flip-flop transfer to the lumen side and is converted to GlcN-acyl-PI (iv) by palmitoylation at C-2 of inositol. Next, three mannoses and two phosphorylethanolamines are added to GlcN-acyl-PI, and then complete GPI anchor binds to a protein at the luminal membrane. The cells defective in the second, third, or fourth reaction [(ii) to (iv)] accumulate GlcNAc-PI, GlcN-PI, or GlcN-acyl-PI, respectively. alk, alkyl or alkenyl; DG, diacylglycerol; PE, phosphatidylethanolamine.



**Fig. 2.** Two-dimensional map of the lipid mixture from CHO mutant cells (M2S2) accumulating GlcNAc-PI obtained by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). LC-ESI-MS using the normal phase column was applied to the lipid mixture extracted from CHO mutants accumulating GlcNAc-PI. The two-dimensional map has  $m/z$  values along the vertical axis and retention times along the horizontal axis. When using a normal phase column, GlcNAc-PIs were eluted before major phospholipids, such as PI, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM).

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,

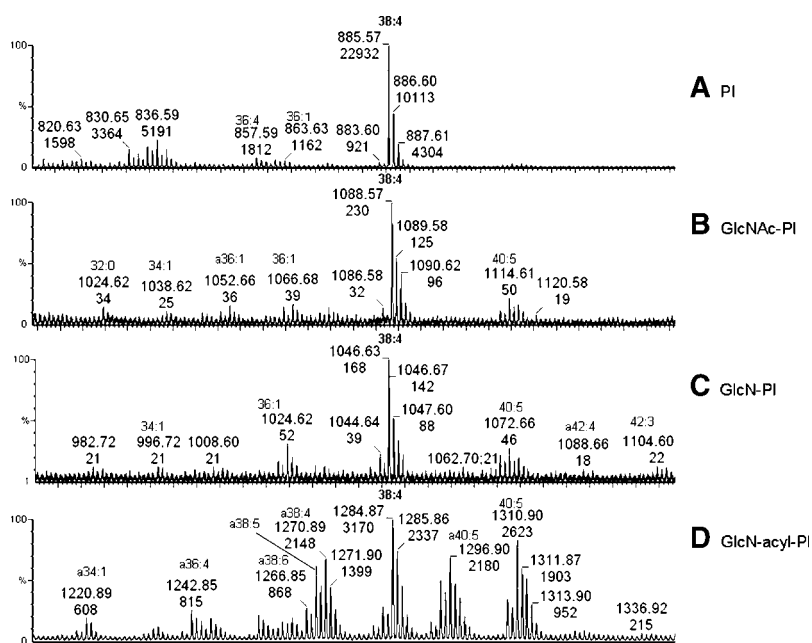
in the fourth precursor (GlcN-acyl-PI), not only 38:4 diacyl species but also many other molecular species, such as 38:4 alk/acyl, were detected as major components (**Fig. 3D**). In this process, molecular species containing longer acyl chain also seem to be accumulated. This result showed that the 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<sup>-</sup>e (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 GPIs 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 peak 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]<sup>-</sup>, C<sub>14</sub>H<sub>23</sub>O<sub>13</sub>NP; **Fig. 5A**); thus, it was confirmed that the peaks 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 (C<sub>18</sub>H<sub>35</sub>O<sub>2</sub>,  $m/z$  283) and 20:4 (C<sub>20</sub>H<sub>31</sub>O<sub>2</sub>,  $m/z$  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 (C<sub>18</sub>H<sub>33</sub>O<sub>2</sub>,  $m/z$  281) [**Fig. 5B, (ii)**].

In **Fig. 6**, the fragment peak at  $m/z$  402 was derived from GlcN-cyclic-inositol phosphate ([GlcN-Ins-P]<sup>-</sup>, C<sub>12</sub>H<sub>21</sub>O<sub>12</sub>NP; **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



**Fig. 3.** Profiles of PI and GPI anchor species in MS spectra of early GPI anchor precursors. Alteration of the molecular species profiles was detected at four early GPI biosynthetic steps. The lipid mixtures from wild-type and GPI mutant cells were investigated by LC-ESI-MS. A: PI. B: GlcNAc-PI. C: GlcN-PI. D: GlcN-acyl-PI.



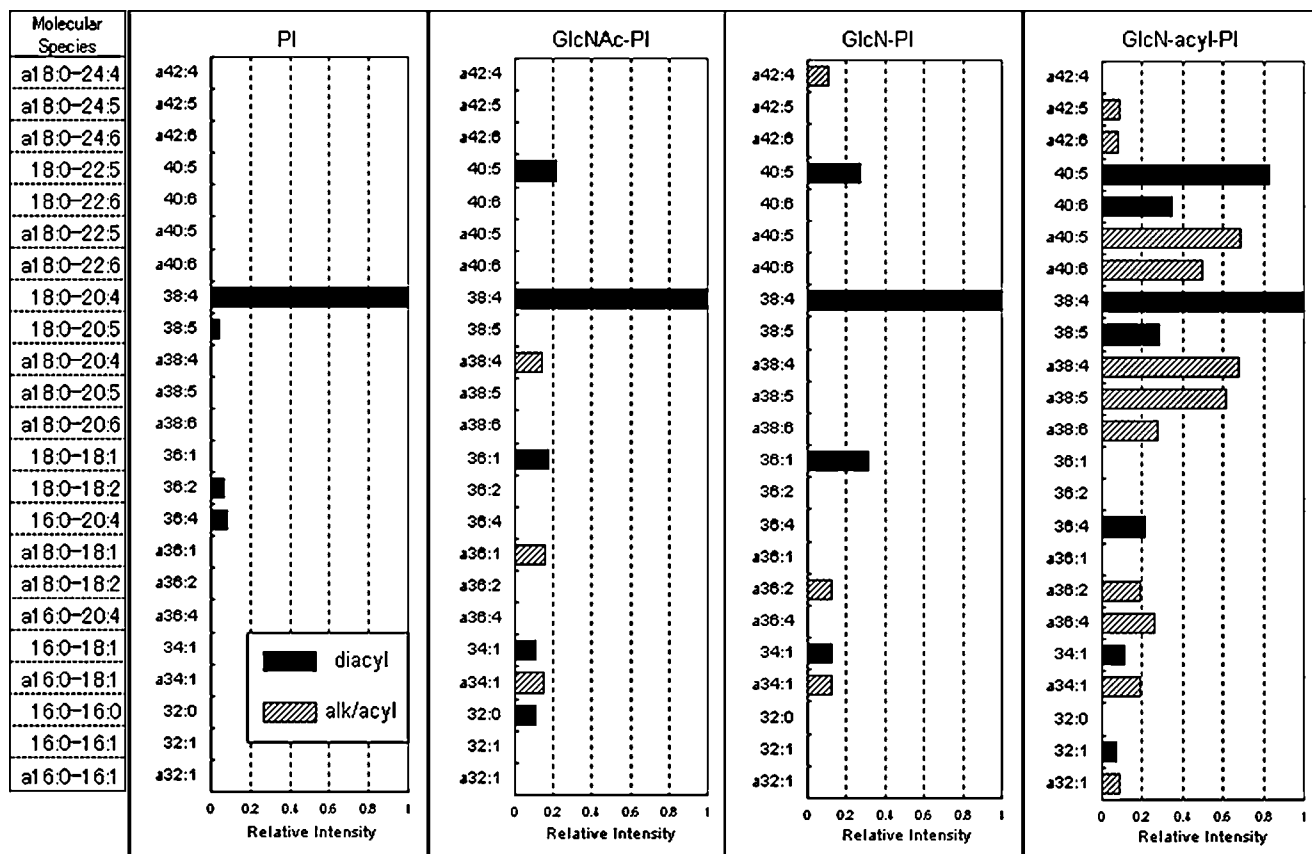


Fig. 4. Comparison of molecular species in PI derivatives of each GPI biosynthetic step. The bar graph compares the intensities of all molecular species at each GPI biosynthetic step relative to the 38:4 (18:0-20:4) diacyl species, which was detected at highest intensity. This graph was created based on the data shown in Fig. 3. Solid and hatched bars indicate diacyl and alk/acyl species, respectively. The table at left shows the major molecular species identified by MS/MS.

the fatty acyl residues 18:0 ( $C_{18}H_{35}O_2$ ,  $m/z$  283) and 22:5 ( $C_{22}H_{33}O_2$ ,  $m/z$  329) and the loss of fatty acyl residue  $[M - f.a.22:5]^-$  ( $C_{33}H_{61}O_{15}NP$ ,  $m/z$  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 ( $C_{18}H_{35}O_2$ ,  $m/z$  283)

and 20:4 ( $C_{20}H_{31}O_2$ ,  $m/z$  303) and  $[M - f.a.20:4]^-$  ( $C_{33}H_{61}O_{15}NP$ ,  $m/z$  742) [Fig. 6B, (ii)].

In Fig. 7, the fragment peak at  $m/z$  640 was derived from GlcN-acyl-cyclic-inositol phosphate ( $[GlcN-Ins-(palmitoyl)-P]^-$ ,  $C_{28}H_{51}O_{13}NP$ ; Fig. 7A); thus, it was confirmed that the

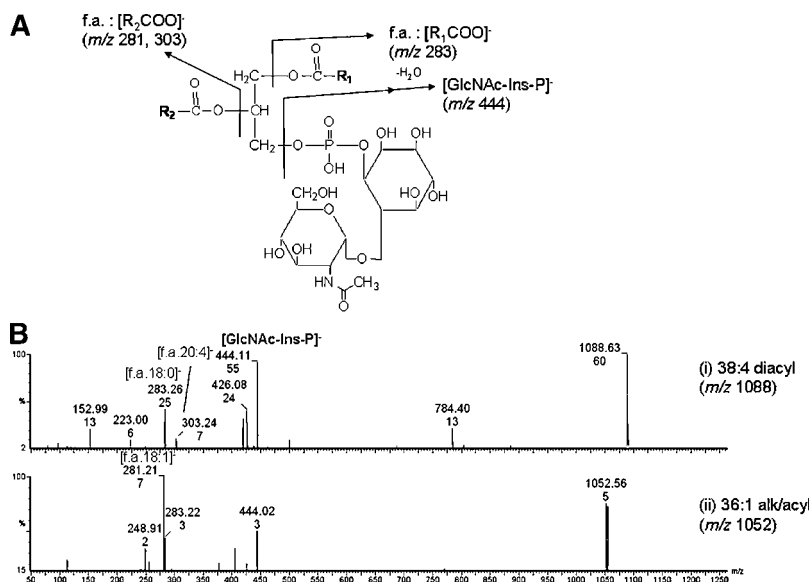
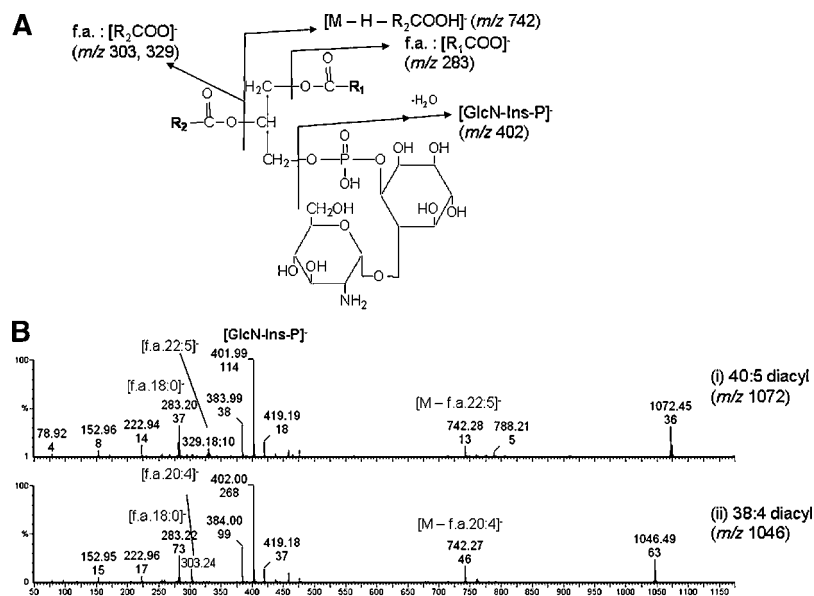


Fig. 5. Structural elucidation of GlcNAc-PI species from specific fragments by LC-ESI-MS/MS. The lipid mixture from CHO mutant cells (M2S2) accumulating GlcNAc-PI was analyzed by LC-ESI-MS/MS. The fragment peak at  $m/z$  444 was identified as the  $[GlcNAc-inositol\ phosphate\ (Ins-P)]^-$  ion, which is derived from the core structure of GlcNAc-PI. A: Fragmentation pattern. B: MS/MS of (i),  $m/z$  1,088 (38:4 diacyl) and (ii),  $m/z$  1,052 (36:1 alk/acyl). f.a., fatty acyl residue.



**Fig. 6.** Structural elucidation of GlcN-PI species from specific fragments by LC-ESI-MS/MS. The lipid mixture from CHO mutant cells (CHOPA10.14) accumulating GlcN-PI was analyzed by LC-ESI-MS/MS. The fragment peak at  $m/z$  402 is the [GlcN-Ins-P]<sup>-</sup> ion, which is derived from the core structure of GlcN-PI. A: Fragmentation pattern. B: MS/MS of (i),  $m/z$  1,072 (40:5 diacyl) and (ii),  $m/z$  1,046 (38:4 diacyl).

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<sub>16</sub>H<sub>31</sub>O<sub>2</sub>,  $m/z$  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<sub>18</sub>H<sub>35</sub>O<sub>2</sub>,  $m/z$  283 and C<sub>20</sub>H<sub>31</sub>O<sub>2</sub>,  $m/z$  303), lyso-phosphatidic acid 18:0 (C<sub>21</sub>H<sub>43</sub>O<sub>7</sub>P,  $m/z$  423), cyclic phosphatidic acid 18:0 (C<sub>21</sub>H<sub>41</sub>O<sub>6</sub>P,  $m/z$  405), and [M - f.a.20:4]<sup>-</sup> (C<sub>49</sub>H<sub>91</sub>O<sub>16</sub>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<sub>16</sub>H<sub>31</sub>O<sub>2</sub>,  $m/z$  255 and C<sub>22</sub>H<sub>31</sub>O<sub>2</sub>,  $m/z$  331) and [M - f.a.22:4]<sup>-</sup> (C<sub>47</sub>H<sub>87</sub>O<sub>16</sub>NP,  $m/z$  952) were detected as fragment ions. Similarly, the peaks at 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]<sup>-</sup> (C<sub>26</sub>H<sub>47</sub>O<sub>13</sub>NP,  $m/z$  612) and a 14:0 fatty acyl residue as fragment ions (C<sub>14</sub>H<sub>27</sub>O<sub>2</sub>,  $m/z$  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).

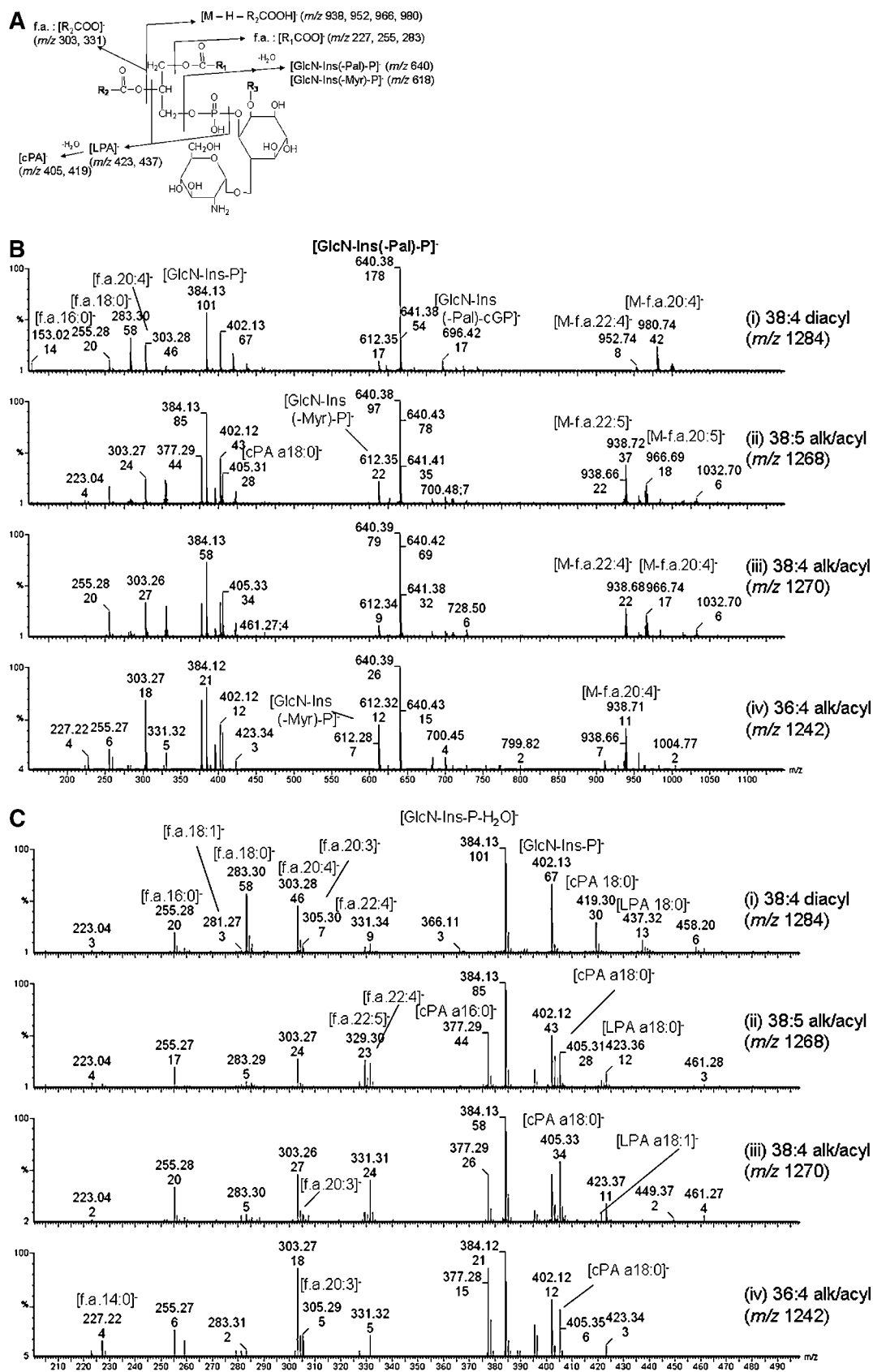
## DISCUSSION

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]<sup>-</sup>;  $m/z$  444) and fatty acyl chain residues (e.g., 18:0-20:4: [f.a. 18:0]<sup>-</sup>;  $m/z$  283 and [f.a. 20:4]<sup>-</sup>;  $m/z$  303) were detected and identified. In the case of diacyl species, two fatty acyl 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 GlcNAc-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 [GlcN-Ins-palmitoyl (Pal)-P]<sup>-</sup> ion, and that at  $m/z$  612 is the [GlcN-Ins-myristoyl (Myr)-P]<sup>-</sup> 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

<i>m/z</i>	Palmitoyl	Myristoyl
1,284	38:4 18:0-20:4 16:0-22:4 18:1-20:3	40:4 18:0-22:4
1,268	a38:5 a18:0-20:5 a18:1-20:4 a16:0022:5	a40:5 a18:0-22:5 a18:1-22:4
1,270	a38:4 a18:0-20:4 a16:0-22:4	a40:4 a18:0-22:4
1,242	a36:4 a16:0-20:4	a38:4 a18:0-20:4 a18:1-20:3 a16:0-22:4

alk/acyl type (30). Together, these results indicate that selection of alkyl/acyl species occurs either in the flip-flop step of GlcN-PI or in the catalytic process of inositol acylation (Fig. 1). In this process, molecular species containing longer acyl chains also seem to be accumulated. Normally, longer fatty acyl (or alkyl) carbon chains have relatively higher hydrophobicity than shorter ones. Also, saturated fatty acyl (or alkyl) chains have relatively higher hydrophobicity than unsaturated ones. Furthermore, fatty alkyl chains have relatively higher hydrophobicity than fatty acyl chains. Thus, alkyl/acyl species have relatively higher hydrophobicity than diacyl species with the same fatty acyl carbon length and the same number of double bonds. If the selection of molecular species could exist in this step, hydrophobicity of substrates might be an important factor. However, other possibilities, such as remodeling of the acyl chain to an alkyl chain at *sn*-1 or substitution of diacyl for alk/acyl diglycerol, cannot be denied. Interestingly, GlcN-acyl-PIs are composed mainly of molecular species with polyunsaturated fatty acyl chains, and saturated chains are rarely present at the *sn*-2 position.

It was shown in two recent reports that fatty acid remodeling of mammalian GPI occurs after GPI is attached to protein (19, 26). Mature GPI-APs on the cell surface are composed mainly of saturated fatty acyl chains, whereas nascent GPI-APs generated in the endoplasmic reticulum contain unsaturated fatty acyl chain at the *sn*-2 position, which is exchanged with stearic acid in the Golgi apparatus during transport to the cell surface. The fatty chain composition of nascent GPI-APs is similar to that of GlcN-acyl-PI shown in this study. Therefore, it seems that GPI anchor precursor that is competent for attachment to protein has a similar fatty chain composition.

Several previous studies demonstrated that acyl chain linked to inositol is heterogeneous in trypanosomes, such as *Trypanosoma brucei* (31, 32) and *T. congolense* (33). In another report, it was found that GlcN-acyl-PI contained myristic acids as minor components (apart from palmitic acids, the major components) at the C-2 position of inositol in malaria parasites (34). It was reported previously that

GlcN-acyl-PI contained only palmitic acid at this position in mammalian cells (30). However, based on our detection of [GlcN-Ins-(myristoyl)-P]<sup>-</sup> fragments (*m/z* 612) (Fig. 7B), we conclude that myristic GlcN-acyl-PIs may also exist. 

The authors thank Ms. Noriko Kagi at Jasco International Co. for quadrupole time-of-flight analysis. This study was performed with the help of Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation.

## REFERENCES

- McConville, M. J., and M. A. Ferguson. 1993. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* **294**: 305–324.
- Ferguson, M. A. 1999. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J. Cell Sci.* **112**: 2799–2809.
- Ikezawa, H. 2002. Glycosylphosphatidylinositol (GPI)-anchored proteins. *Biol. Pharm. Bull.* **25**: 409–417.
- Homans, S. W., M. A. Ferguson, R. A. Dwek, T. W. Rademacher, R. Anand, and A. F. Williams. 1988. Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. *Nature.* **333**: 269–272.
- Proudfoot, L., P. Schneider, M. A. Ferguson, and M. J. McConville. 1995. Biosynthesis of the glycolipid anchor of lipophosphoglycan and the structurally related glycoinositolphospholipids from *Leishmania major*. *Biochem. J.* **308**: 45–55.
- Taguchi, R., N. Hamakawa, M. Harada-Nishida, T. Fukui, K. Nojima, and H. Ikezawa. 1994. Microheterogeneity in glycosylphosphatidylinositol anchor structures of bovine liver 5'-nucleotidase. *Biochemistry.* **33**: 1017–1022.
- Brown, D. A., and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* **68**: 533–544.
- Harder, T., and K. Simons. 1997. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr. Opin. Cell Biol.* **9**: 534–542.
- Tansey, M. G., R. H. Baloh, J. Milbrandt, and E. M. Johnson, Jr. 2000. GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron.* **25**: 611–623.
- Sabharanjak, S., P. Sharma, R. G. Parton, and S. Mayor. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell.* **2**: 411–423.
- Kinoshita, T., and N. Inoue. 2000. Dissecting and manipulating the pathway for glycosylphosphatidylinositol-anchor biosynthesis. *Curr. Opin. Chem. Biol.* **4**: 632–638.
- Miyata, T., J. Takeda, Y. Iida, N. Yamada, N. Inoue, M. Takahashi, K. Maeda, T. Kitani, and T. Kinoshita. 1993. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. *Science.* **259**: 1318–1320.
- Leidich, S. D., D. A. Drapp, and P. Orlean. 1994. A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. *J. Biol. Chem.* **269**: 10193–10196.
- Takeda, J., T. Miyata, K. Kawagoe, Y. Iida, Y. Endo, T. Fujita, M. Takahashi, T. Kitani, and T. Kinoshita. 1993. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell.* **73**: 703–711.
- Homans, S. W., M. A. Ferguson, R. A. Dwek, T. W. Rademacher, R. Anand, and A. F. Williams. 1988. Complete structure of the glycosyl phosphatidylinositol membrane anchor of rat brain Thy-1 glycoprotein. *Nature.* **333**: 269–272.
- Roberts, W. L., J. J. Myher, A. Kuksis, and T. L. Rosenberry. 1988. Alkylacylglycerol molecular species in the glycosylated phospholipid membrane anchor of bovine erythrocyte acetylcholinesterase. *Biochem. Biophys. Res. Commun.* **150**: 271–277.
- Brewis, I. A., M. A. Ferguson, A. Mehlert, A. J. Turner, and N. M. Hooper. 1995. Structures of the glycosyl-phosphatidylinositol anchors of porcine and human renal membrane dipeptidase. Com-



- prehensive structural studies on the porcine anchor and interspecies comparison of the glycan core structures. *J. Biol. Chem.* **270**: 22946–22956.
18. Treumann, A., M. R. Lifely, P. Schneider, and M. A. Ferguson. 1995. Primary structure of CD52. *J. Biol. Chem.* **270**: 6088–6099.
19. Tashima, Y., R. Taguchi, C. Murata, H. Ashida, T. Kinoshita, and Y. Maeda. 2006. PGAP2 is essential for correct processing and stable expression of GPI-anchored proteins. *Mol. Biol. Cell.* **17**: 1410–1420.
20. Kerwin, J. L., A. R. Tuininga, and L. H. Ericsson. 1994. Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry. *J. Lipid Res.* **35**: 1102–1114.
21. Han, X., and R. W. Gross. 1994. Electrospray ionization mass spectroscopic analysis of human erythrocyte plasma membrane phospholipids. *Proc. Natl. Acad. Sci. USA.* **91**: 10635–10639.
22. Nakamura, N., N. Inoue, R. Watanabe, M. Takahashi, J. Takeda, V. L. Stevens, and T. Kinoshita. 1997. Expression cloning of PIG-L, a candidate N-acetylglucosaminyl-phosphatidylinositol deacetylase. *J. Biol. Chem.* **272**: 15834–15840.
23. Murakami, Y., U. Siripanyapinyo, Y. Hong, J. Y. Kang, S. Ishihara, H. Nakakuma, Y. Maeda, and T. Kinoshita. 2003. PIG-W is critical for inositol acylation but not for flipping of glycosylphosphatidylinositol-anchor. *Mol. Biol. Cell.* **14**: 4285–4295.
24. Maeda, Y., S. Tomita, R. Watanabe, K. Ohishi, and T. Kinoshita. 1998. DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: correct subcellular localization and stabilization of DPM1, and binding of dolichol phosphate. *EMBO J.* **17**: 4920–4929.
25. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Med. Sci.* **37**: 911–917.
26. Maeda, Y., Y. Tashima, T. Houjou, M. Fujita, T. Yoko-O, Y. Jigami, R. Taguchi, and T. Kinoshita. 2007. Fatty acid remodeling of GPI-anchored proteins is required for their raft association. *Mol. Biol. Cell.* **18**: 1497–1506.
27. Jungalwala, F. B., J. E. Evans, and R. H. McCluer. 1984. Compositional and molecular species analysis of phospholipids by high performance liquid chromatography coupled with chemical ionization mass spectrometry. *J. Lipid Res.* **25**: 738–749.
28. Kakela, R., P. Somerharju, and J. Tyynela. 2003. Analysis of phospholipid molecular species in brains from patients with infantile and juvenile neuronal-ceroid lipofuscinosis using liquid chromatography-electrospray ionization mass spectrometry. *J. Neurochem.* **84**: 1051–1065.
29. Houjou, T., K. Yamatani, M. Imagawa, T. Shimizu, and R. Taguchi. 2005. A shotgun tandem mass spectrometric analysis of phospholipids with normal-phase and/or reverse-phase liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**: 654–666.
30. Sevelev, D., D. R. Humphrey, and T. L. Rosenberry. 1995. Compositional analysis of glucosaminyl(acyl)phosphatidylinositol accumulated in HeLa S3 cells. *Eur. J. Biochem.* **233**: 384–394.
31. Guther, M. L., A. Treumann, and M. A. Ferguson. 1996. Molecular species analysis and quantification of the glycosylphosphatidylinositol intermediate glycolipid C from *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **77**: 137–145.
32. Treumann, A., N. Zitzmann, A. Hulsmeier, A. R. Prescott, A. Almond, J. Sheehan, and M. A. Ferguson. 1997. Structural characterisation of two forms of procyclic acidic repetitive protein expressed by procyclic forms of *Trypanosoma brucei*. *J. Mol. Biol.* **269**: 529–547.
33. Thomson, L. M., D. J. Lamont, A. Mehlert, J. D. Barry, and M. A. Ferguson. 2002. Partial structure of glutamic acid and alanine-rich protein, a major surface glycoprotein of the insect stages of *Trypanosoma congolense*. *J. Biol. Chem.* **277**: 48899–48904.
34. Naik, R. S., O. H. Branch, A. S. Woods, M. Vijaykumar, D. J. Perkins, B. L. Nahlen, A. A. Lal, R. J. Cotter, C. E. Costello, C. F. Ockenhouse, et al. 2000. Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. *J. Exp. Med.* **192**: 1563–1576.